Functional Analyses of the role of kisspeptins and their receptor, gpr-54 in the biology of reproductive tissues

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Declaration

I hereby declare that the work presented in this thesis was carried out by myself during the course of my PhD and has not been submitted for any other degree or qualification. Where I have used the work of others, the sources of the information have been detailed explicitly in the presentation.

Antonia Roseweir

Acknowledgments

Firstly, I would like to give my thanks to my supervisors, Robert Millar and Kevin Morgan for all of their help throughout my PhD. Bob for always keeping my writing skills up to scratch and for help designing the peptide analogues and Kevin for knowing the answer to almost all of my questions about my research and science in general and for critically reading this thesis. To all the other members of the Bob Millar group for their support, friendship and advice, especially the technicians, who's technical and cell culture support has been invaluable. I would especially like to thank Nicola for teaching me many techniques and helping with assays when I was about to explode.

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I would also like to thank my mum and dad for great support and financial help throughout my PhD. I would especially like to thank my husband, Jason for his support throughout this research and for putting up with me when things weren't going to plan. Finally, I would like to thank my cats for always being there to give me a cuddle.

Publications and Presentations

The majority of the research in chapter three has been published in the Journal of Neuroscience:

Roseweir A.K., Kauffman A.S., Smith J.T., Guerriero K.A., Morgan K., Pielecka-Fortuna J., Pineda R., Gottsch M.L., Tena-Sempere M., Moenter S.M., Terasawa E., Clarke I.J., Steiner R.A., and Millar R.P., *Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation* J Neuroscience, 2009. **29**(12): p 3920-3929.

I was also successful in publishing a first author review in Human Reproduction Update and a third author review in Reproduction:

Roseweir A.K. and Millar R.P., *The role of kisspeptins in the control of gonadotrophin secretion* Human Reproduction Update, 2009. **15**(2): p203-212.

Reynolds R.M., Logie J.J., **Roseweir A.K.**, McKnight A.J. and Millar R.P., *A Role for kisspeptin in pregnancy: facts and speculations* Reproduction, 2009. **138** (1): p1-7.

The research within this thesis was also presented via oral presentations or posters at the Biochemical Society Meeting called Lifesciences 2007 in Glasgow, UK, at the 90th Endocrine Society Meeting in San Francisco, USA in 2008, at the 1st International Conference for Kisspeptin Signalling in the Brain in Cordoba, Spain in 2008, at the British Endocrine Society Meeting in Harrogate, UK in 2009, at the Society for Gynaecological Investigation Annual Conference in Glasgow, UK in 2009, at the British Society for Neuroendocrinology Meeting in Edinburgh, UK in 2009 and at the British Pharmacological Society Summer Meeting in Edinburgh, UK in 2009.

Hypothesis

- Kisspeptin and gpr-54 regulate the HPG axis via GnRH.
- Kisspeptin and gpr-54 are critical to puberty onset.
- Kisspeptin signals via differential mechanisms in GnRH neuronal and trophoblast cell lines.
- Kisspeptin modulates migratory signalling pathways in model and placental cell lines.
- Kisspeptin regulates migration of a model cell line and trophoblast cells in the placenta.

Aims

- To develop and characterise kisspeptin analogues to screen for agonists and antagonists.
- To test antagonists *in vitro* and *in vivo* to delineate the role of kisspeptin in the HPG axis and puberty.
- To investigate the signalling pathways activated by kisspeptin-10 in a model cell line (CHO/gpr-54), GnRH neuronal cell line, placental cell lines and cancer cell lines.
- To determine the signalling pathways important for kisspeptin-mediated inhibition of migration.

Abstract

GnRH neurons represent the final common pathway for the regulation of the reproductive axis and they are modulated by multiple signals. It has recently been shown that a potent effector of GnRH neuron function is an afferent network of kisspeptin-producing neurons. Kisspeptin released from these neurons acts upon a specific receptor (gpr-54) expressed on GnRH neurons, and increases the secretion of GnRH from the hypothalamus. The kisspeptin system has since been implicated as a downstream mediator for regulation of the Hypothalamic-Pituitary-Gonadal (HPG) axis by steroid hormones, metabolic signals and photoperiod, potentially placing it at the centre of reproductive physiology. However, the supporting evidence to date has been indirect, relying on interpretation of changes in mRNA levels and immuno-histochemical staining to infer the actions of kisspeptin upon the central control of reproduction. The detailed mechanisms of kisspeptin action are yet to be fully elucidated.

The research within this thesis elucidates the effect of kisspeptin on the HPG axis via the development of kisspeptin-10 (kp-10) analogues with antagonistic properties. Functionally important residues within the peptide were delineated. Structure-activity studies of kp-10 analogues indicated that residues Asn^2, Trp^3, Phe^6, Arg^9 and Phe^{10} interact with gpr-54 to facilitate receptor binding. Two other residues, Tyr^1 and Leu^8 were shown to be critical for receptor activation by kisspeptin. Four synthetic peptide antagonists were selected according to a consensus sequence for good antagonism: X^1-N-W-N-X^5-F-G-X^8-R-F-NH_2 where X^1 = D-Ala or D-Tyr, X^5 = Gly or D-Ser and X^8 = D-Trp or D-Leu. One of the antagonists, peptide 234, was used in *in vivo* studies, where it inhibited the amplitude of GnRH and LH pulses without affecting basal secretion of GnRH or LH. These results indicate for the first time that basal and pulsatile secretion of these factors is regulated by separate pathways. Use of the antagonist also

demonstrated the direct involvement of endogenous kisspeptin in steroid hormone negative feedback, positive regulation of the pre-ovulatory LH surge and in regulating the onset of puberty in rodents, as had been suggested via indirect methods.

Although a major role of the kisspeptin system is in the regulation of the HPG axis, the system may also be important in the inhibition of cancer cell metastasis and in placental development (trophoblast cell invasion) but little is known about the mechanisms involving kisspeptin in these processes. This thesis describes novel signalling mechanisms for the regulation cell migration by kisspeptin, involving the MAPK and GSK3β signalling pathways. Using a stably transfected CHO cell line, kisspeptin-gpr-54 signalling can activate all members of the MAPK pathway, the β-catenin/GSK3β pathway, NFκB and FAK. These factors are involved in inhibiting the migration of these cells via an ERK1/2-p90rsk-GSK3β-β catenin pathway to potentially up- regulate formation of adherens junctions at the plasma membrane. This pathway was also shown to be involved in the inhibition of migration within an immortalised human first trimester placental trophoblast cell line and in human umbilical vein endothelial cells. Some of these pathways were also active within a mouse GnRH neuronal cell line, where ERK1/2, NFκB and GSK3β were activated by kisspeptin with no effect on migration. However, the role of these pathways in the GnRH neuronal cells requires further investigation.

In summary, the research presented within this thesis defines receptor-binding and activating residues within kisspeptin-10, which should enable more details of ligand-receptor binding interactions to be fully elucidated. Novel gpr-54 antagonists have been identified and used in *in vivo* studies. The thesis demonstrates the direct involvement of endogenous kisspeptin in the regulation of GnRH/LH secretion at the onset of puberty and throughout the reproductive cycle in mature animals. The antagonists developed within this thesis represent useful tools to further delineate mechanisms of kisspeptin action within the HPG axis and

peripheral tissues. Other findings describe kisspeptin signalling mechanisms for the inhibition of cell migration, potentially important in a variety of normal and pathological processes, including for the first time a description of the regulation of GSK3β and β-catenin signalling factors by kisspeptin and gpr-54.

Chapter One

Literature Review

1.0. Introduction

This literature review will cover advances in our understanding of the role of kisspeptin and gpr-54 in reproduction. It will focus on kisspeptin and gpr-54 as major regulators of the hypothalamic-pituitary-gonadal axis, their role in puberty, steroid hormone feedback and metabolic regulation of reproduction. It will also review putative functions of the kisspeptin/gpr-54 system in peripheral reproductive systems, such as in the placenta where a role in inhibiting trophoblast invasion has been proposed and in the ovary where kisspeptin may directly regulate ovulation. This literature review will also cover the signalling pathways which have been implicated in kisspeptin/gpr-54 actions and the specific signalling pathways examined within this thesis.

1.1. Discovery of kisspeptin and gpr-54

1.1.1. Discovery of kisspeptins and RFamide peptide hormones

Kisspeptins are peptide products of the *KiSS-1* gene, which was first discovered by Lee et al. in 1996 as a metastasis suppressing gene in malignant melanoma cells (Lee et al., 1996). As it was discovered in Hershey, USA, the gene was named after Hershey's famous chocolate kisses; however, the nomenclature also has a scientific grounding as the inclusion of SS in the name also indicates that the gene is a cancer suppressor sequence. The *KiSS-1* gene is located on human chromosome 1q32 and consists of 2 non-translated and 2 partially translated regions within 4 exons to give rise to an 145 amino acid precursor peptide (West et al., 1998). This precursor is then cleaved to 54 amino acids in length, which can be further truncated to 14, 13 or 10 amino acid carboxyl terminal fragments (Fig. 1). Collectively these N-terminally truncated peptides are known as the kisspeptins, and belong to a larger family of peptides known as RFamides which all share a common Arg-Phe-NH_2 motif at their C-terminus.

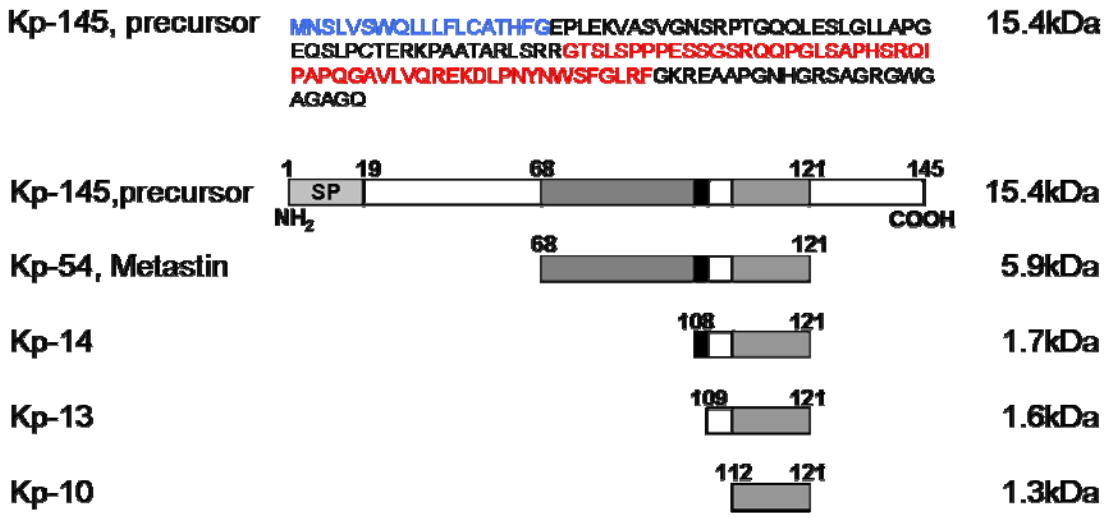

Figure 1. Kisspeptin carboxyl terminal fragments. Schematic diagram showing the relative size and cleavage points for processing of human kisspeptin-145 (Kp-145) to the carboxyl terminal fragments, Kp-54, Kp-14, Kp-13 and Kp-10. Also shown is the signal peptide (SP) within the Kp-145 precursor peptide. The amino acid sequence for human kp-145 is given with kp-54 (red) and SP (blue) highlighted.

RFamide hormones were first discovered in 1977 when FMRFamide was isolated from the clam, *Macrocallista nimbosa,* as a cardioexcitatory peptide. Since then RFamides have been found in many vertebrates and invertebrates, with the first mammalian RFamides discovered in 1985. The two peptides, Neuropeptide FF (NPFF) and neuropeptide AF (NPAF), were found in bovine brain extracts using an anti-sera to the original RFamide (FMRFamide), and have subsequently been shown to be derived from a single gene. Since then, due to a breakthrough in pharmacological methods for identifying ligands for orphan g-protein coupled receptors (GPCRs), many more mammalian RFamides have been discovered (Table 1). In fact it is by this method that the kisspeptin peptides were found to act on an orphan GPCR. As more RFamides such as kisspeptins are discovered, it can be seen that many of these peptides have important functions in reproductive systems. NPSF and NPFF have been reported to inhibit

release of gonadotropins in mammals and birds and kisspeptin has been shown to stimulate gonadotropins (Fukusumi et al., 2006). Kisspeptins are highly conserved within mammalian (Clements et al., 2001; Kotani et al., 2001) and non-mammalian vertebrates (Biran et al., 2008; Kanda et al., 2008; van Aerle et al., 2008), suggesting they have an important role to play in a wide range of animals.

Peptide Gene	Peptide Name	Receptor Gene	Receptor Name
farp-1	NPAF	*rfr-3*	GPR74/NPGPR/HLWAR77/NPFF-2
	NPFF		GPR147/NPFF-1/OT7T022
farp-2	PrPR	*rfr-1*	GPR10/hGR3/UHR-1
farp-3	RFRP-1/NPSF	*rfr-2*	GPR147/NPFF-1/OT7T022
	RFRP-3/NPVF		GPR74/NPGPR/HLWAR77/NPFF-2
farp-4	Kisspeptins	*rfr-4*	GPR54/OT7T175/AXOR12/KISS-1R
farp-5	26RFa/P513/QRFP	*rfr-5*	GPR103/AQ27/SP9155

Table 1. Summary of mammalian RFamides and their receptors. The designations of the peptide-encoding genes (*farp-1* to -5) and their receptor genes (*rfr-1* to -5) are proposed by Dockray (Dockray, 2004). Alternative names for peptides and receptors are indicated by '/'. Table taken from Fukusumi et al., *Recent advances in mammalian RFamide peptides: The discovery and functional analyses of PrPR, RFRPs and QRFP*. Peptides 2006. **27** (5): p1073-1086.

1.1.2. Discovery of gpr-54

Kisspeptins are the natural ligands for the orphan GPCR known as gpr-54 in rat (Lee et al., 1999) and AXOR12 in humans (Muir et al., 2001). However, for ease, throughout the rest of the thesis only the gpr-54 designation will be used. Gpr-54 is a 396 amino acid receptor and is a member of the rhodopsin family/class A g-protein coupled receptors (Fig. 2). Gpr-54 was first discovered in 1999 by a PCR search of rat brain, as an orphan receptor related to galanin receptors (GalR). Gpr-54 had a 45% homology to

GalR1-3 (Fig. 3); however it possessed a relatively low affinity for the ligand, galanin (Lee et al., 1999). Then in 2001, two papers reported that peptides derived from the *KiSS-1* gene (kisspeptins) were the natural ligands for gpr-54 (Kotani et al., 2001; Muir et al., 2001). Muir et al. used a reverse pharmacology system, screening a large library of possible ligands for calcium mobilization (Muir et al., 2001). Whereas, Kotani et al. purified fractions from tissue extracts where gpr-54 was known to be expressed. These were then tested on an aequorin-based luminescence assay and two hplc peaks from a placental extract where purified and sequenced. The sequence and mass was then compared to protein and nucleotide sequence libraries to identify kisspeptin-13 and kisspeptin-10 (Kotani et al., 2001). Studies also discovered, that gpr-54 is highly conserved among mammals with the human receptor having 85% homology to the rat receptor, 80% homology to the mouse (Clements et al., 2001) and about 40% homology to non-mammalian vertebrates, such as bullfrogs (Moon et al., 2008) .

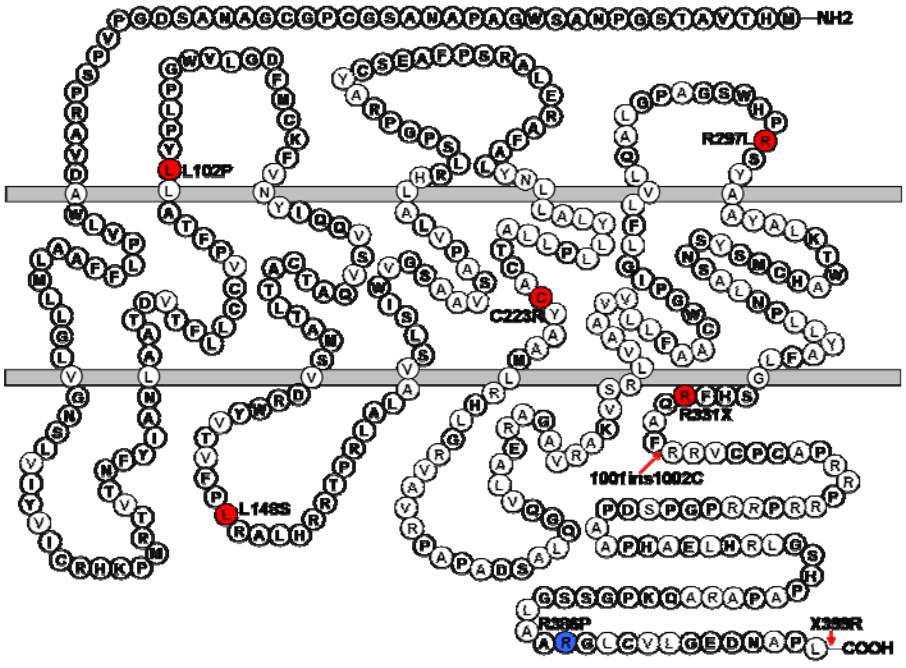

Figure 2. Schematic diagram of human gpr-54. Diagram showing amino acid sequence of human gpr-54. The diagram shows the seven transmembrane structure of the receptor, with an extracellular N-terminus and cytoplasmic C-terminus. Inactivating mutations (red) and activating mutations (blue) are highlighted.

The 10 amino acid carboxyl-terminal sequence of mature kisspeptin (kp-10) is the minimal length required for activation of gpr-54. The receptor couples to the $G_{q/11}$ class of g-proteins to activate phospholipase C (PLC) which hydrolyses phosphatidyl inositol bisphosphate (PIP_2) in the cell membrane to diacyl glycerol (DAG) to activate protein kinase C (PKC) and inositol trisphosphate (IP_3) which modulates release of intracellular calcium. The activation of PKC then leads to phosphorylation of ERK1/2 and p38MAPK, reorganisation of intracellular stress fibres and induction of focal adhesion kinase to inhibit cell movement (Fig. 4), a process that is thought to be important for kisspeptin-mediated suppression of cancer metastasis. (Hori et al., 2001; Kotani et al., 2001). Kisspeptin can also inhibit the migration and invasive capacity of a model CHO cell line stably expressing the gpr-54 receptor, further suggesting a role for the system in the regulation of cell migration (Hori et al., 2001).

1.1.3. Reproductive functions for kisspeptin and gpr-54

Although kisspeptins and gpr-54 were first described in relation to cancer metastasis, kisspeptin action was subsequently shown to play a pivotal role in the control of the hypothalamic-pituitary-gonadal (HPG) axis via regulation of gonadotropin-releasing hormone (GnRH) secretion (Funes et al., 2003; Gottsch et al., 2004; Irwig et al., 2004; Messager et al., 2005). Immunohistochemical localisation and mRNA analysis of the receptor and ligand within the brain and peripheral tissues, demonstrated gpr-54 and *KiSS-1* expression within the hypothalamus, brainstem, spinal cord, pituitary, ovary, prostate and placenta. This localisation suggests they may regulate the reproductive axis at a number of levels (Castellano et al., 2006a; Clements et al., 2001; Kotani et al., 2001; Mead et al., 2006; Muir et al., 2001). The high expression in the placenta, led researchers to hypothesise that there may be a role for kisspeptin/gpr-54 in placentation. Then in 2004, kisspeptin was shown to inhibit the migration of primary trophoblast explants, supporting this hypothesis (Bilban et al., 2004).

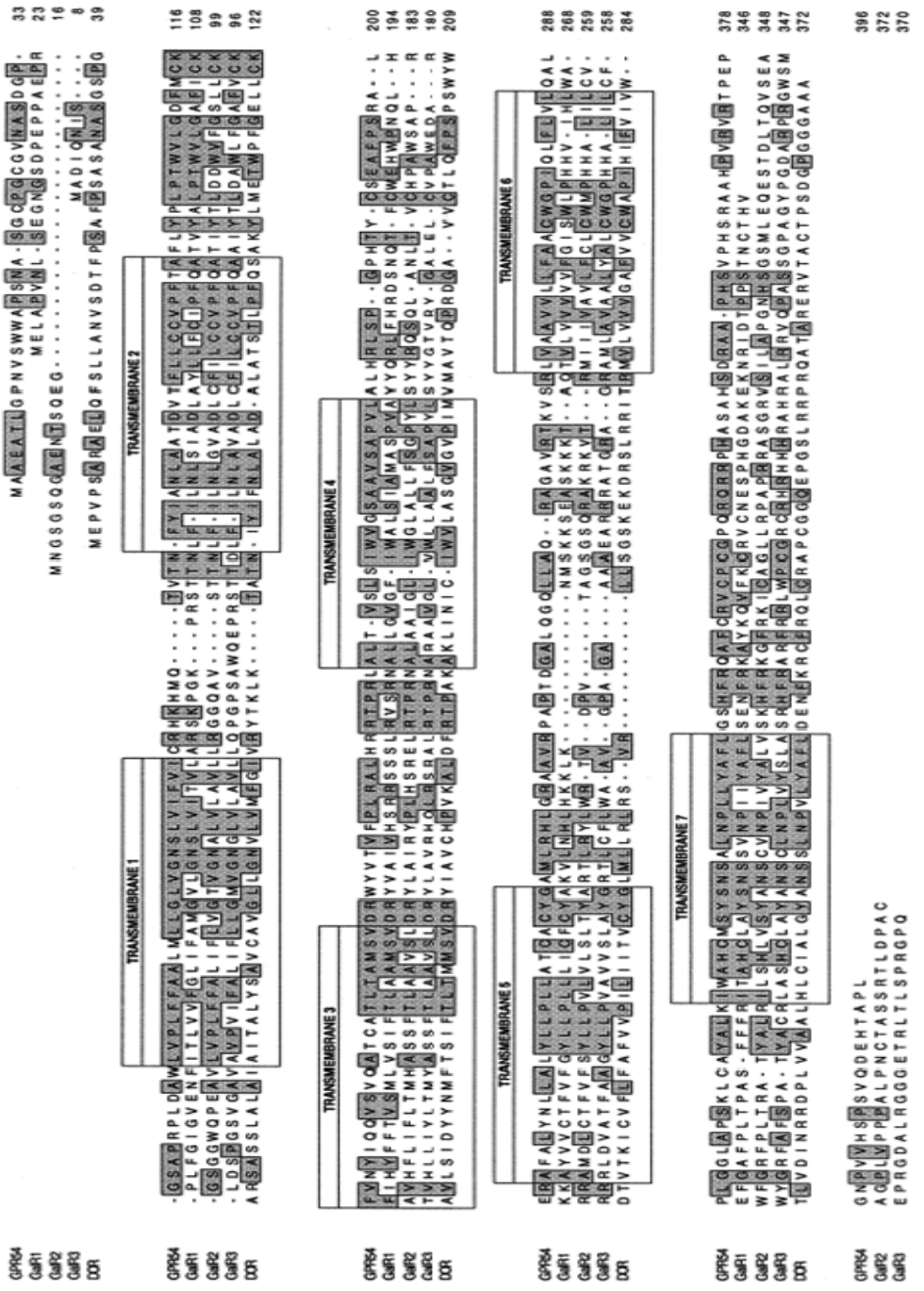

Figure 3. Gpr-54 and the Galanin receptors. Schematic alignment of Gpr-54 and GalR1-3 with conserved residues indicated by the shaded boxes. The schematic also highlights the seven transmembrane domains. Diagram taken from Lee et al. *Discovery of a receptor related to the galanin receptors* FEBS letters 1999. **446** (1): p103-107.

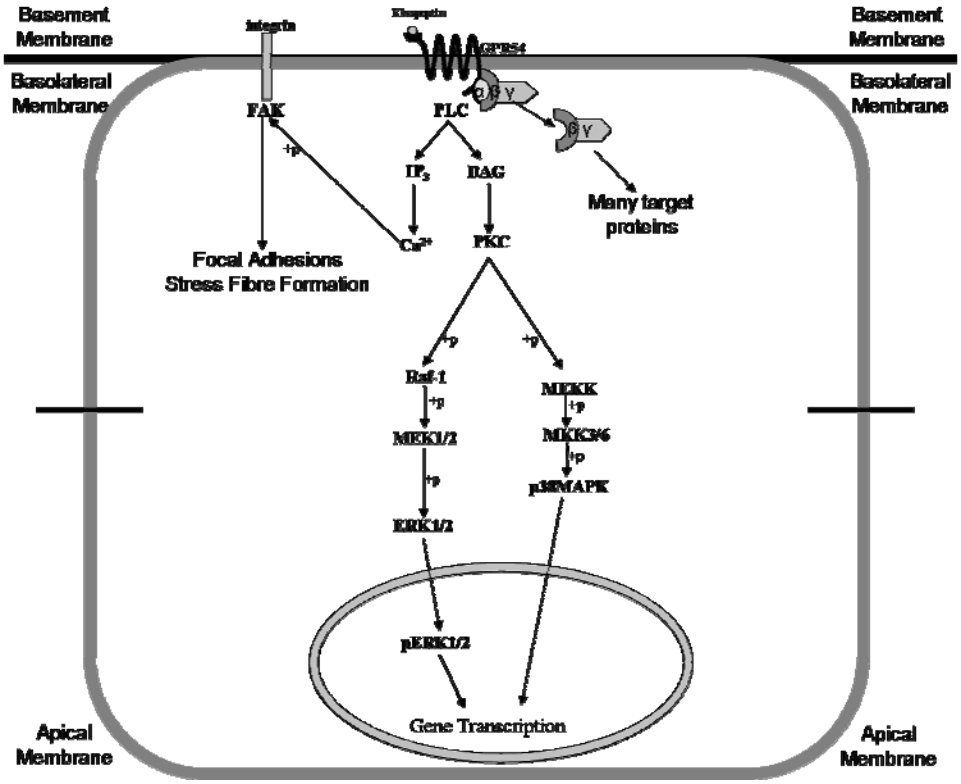

Figure 4. Primary signalling pathways activate by Gpr-54 via $G\alpha_{q/11}$. Signalling diagram showing gpr-54 activating the classical $G\alpha_{q/11}$ signalling pathway in an epithelial cell. This activates inositol phosphate to stimulate intracellular calcium release and diacyl glycerol (DAG) to activate PKC. Activation of the MAPK and focal adhesion pathways has also been shown.

1.2. Structure of KiSS-1, kisspeptins and gpr-54

1.2.1. Structural elements of the KiSS-1 gene

The human *KiSS-1* gene was first discovered in 1996 and has since been mapped to the long arm of chromosome 1 at 1q32 by radiation hybrid mapping and fluorescent in-situ hybridisation (FISH). This has been confirmed by genome sequencing (see Ensemble database). The gene consists of four exons of which two are non-translated and two translated exons (Fig. 5), which give rise to an open reading frame (ORF) of 438 base pairs (bp) or 145 amino acids. The first exon consists of 109 non-coding base pairs and

exon 2 contains 91 non-coding bases. Translation commences in the third exon where 35 non-coding base pairs are followed by a putative methionine start codon and 103 translated base pairs. The fourth exon is the largest and contains 335 base pair long coding region and a stop codon followed by 121 non-coding bases, which contains a polyadenylation sequence (West et al., 1998). Two introns have also been partially identified; one is thought to be 37 bases upstream of the methionine start codon and the second around base 103 of the coding region. Analysis of the sequence also found the following homology domains: a tyrosine kinase phosphorylation site was located within amino acid positions 105-112 with a sequence of REKDLPNY. An N-myristoylation site was also found at amino acids 118-123; this had the sequence GLRFGK. Two PKC consensus sites were also found within *KiSS-1* at amino acid positions 54-56 and 61-63. These sequences followed the motif [S/T]-X-[R/K] and finally a PKA phosphorylation site was located at amino acids 66-69 with the motif $[R/K]_2$-X-[S/T]. This suggests that the *KiSS-1* gene is modified at a number of sites to effect translation and post-translational regulation (Lee et al., 1999; West et al., 1998).

Figure 5. Schematic diagram of *KiSS-1* gene. Diagram showing intron and exon lengths, with the ORF for kisspeptin pre-peptide shown in black.

1.2.2. Structural elements of kisspeptins

The *KiSS-1* gene encodes a polypeptide consisting of 145 amino acids, known as the precursor peptide to kisspeptins. This precursor peptide contains a 19 amino acid leader sequence that directs the protein into the endoplasmic reticulum (ER) and which gives rise to a secretory precursor protein of 126 amino acids which is proteolytically cleaved and modified to form a C-terminal amide moiety in the Golgi membrane network

(Takino et al., 2003). The kisspeptin-54 (metastin) produced is then cleaved further to smaller fragments of 14, 13 and 10 amino acids in length. These fragments have subsequently been shown to bind to and activate gpr-54 with equal potency (Kotani et al., 2001). Therefore, the final 10 amino acid fragment of the C-terminus has been used to analyse the structure of kisspeptins by nuclear magnetic resonance spectroscopy (NMR). Two studies have used NMR to determine the structure; the first used sodium dodecyl sulphate (SDS) micelles NMR, which involves use of a negatively charged membrane. In this study kisspeptin-13 was utilised and residues Asn^{7} to Phe^{13} were found to form a stable helical structure (Orsini et al., 2007). The second study utilised dodecylphosphocholine (DPC) micelles NMR which represent a membrane with a neutral charge, similar to the mammalian lipid membrane. Using kisspeptin-10, they showed that Trp^{3} to Phe^{10} contained several tight turn structures but no helices. The tight turns contained one β-turn from Tyr^{3} to Phe^{6}; two miscellaneous type IV β-turns at Asn^{4} to Gly^{7} and Gly^{7} to Phe^{10}; and finally one γ-turn from Ser^{5} to Gly^{7} (Lee et al., 2009). This suggests that hydrophobic environments may cause kisspeptins to form a more ordered structure allowing them to bind and activate gpr-54 (Fig. 6).

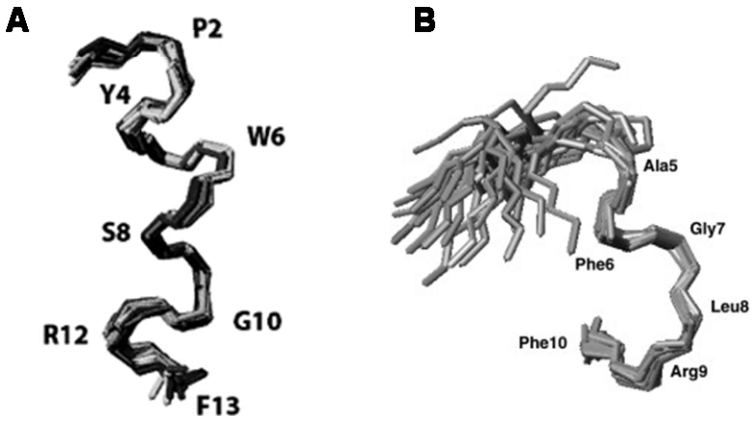

Figure 6. NMR structures for Kisspeptins. (A) NMR model from Orsini et al. (Orsini et al., 2007) showing helical structure of kisspeptin-13. (B) NMR model from Lee et al. (Lee et al., 2009) showing alternative tight turn structure for kisspeptin-10.

The use of NMR to determine the structure of kisspeptin fragments has also shown that hydrogen bonds are important within these peptides, with eight hydrogen bonds being formed within Kisspeptin-13, allowing 16 hydrogen constraints. These are formed between residues Phe[9], Leu[11], Arg[12] and Phe[13]. These four residues have also been shown to form a pharmacophore which is important for receptor binding. The pharmacophore consists of the phenyl ring of Phe[9] with the Phe[13] phenyl ring on top. These are then flanked by the positively charged Arg[12], which is all in close proximity to the amide moiety (Fig. 7). Even though the pharmacophore residues can form hydrogen bonds, it appears that the positive charge is more important for receptor binding (Orsini et al., 2007).

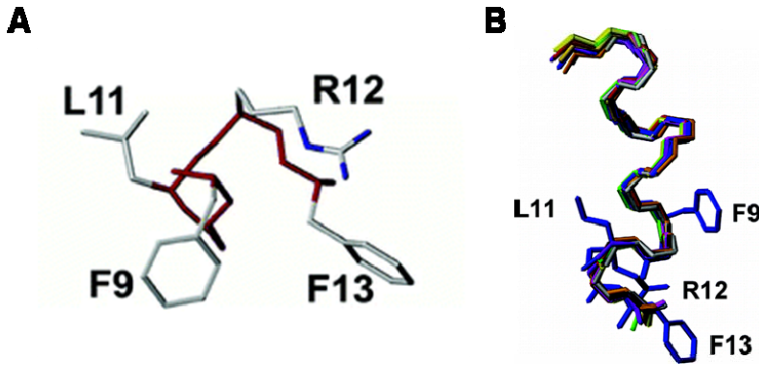

Figure 7. Binding pharmacophore within Kisspeptin-10. (A) Diagram of binding pharmacophore alone showing the two phenyl rings on top of each other, next to the positively charged arginine. (B) Model showing position of pharmacophore within the helical NMR structure of kisspeptin-13. Diagrams taken from Orsini et al. *Metastin (KISS-1) mimetics identified from peptide structure-activity relationship-derived pharmacophores and direct small molecule database screening* J Med Chem 2007. **50**(3): p462-471.

All kisspeptins fragments have also been shown to contain a matrix metalloproteinase (MMP) mediated cleavage site, which is thought to be a mechanism to inactivate the protein. This site is at the C-terminus consisting of Phe-Gly-Leu-Arg, where MMP-2 and MMP-9 can cleave kisspeptin-54 between Gly[51] and Leu[52]. MMPs have also been shown to form a complex with the kisspeptin precursor peptide involving the N-terminal 48 amino acids with Cys[53] being critical to this process and the propeptide domain of MMPs (Takino et al., 2003).

1.2.3. Structural elements of gpr-54

Gpr-54 is a member of the rhodopsin-like or class A family of g-protein coupled receptors located on human chromosome 19p13.3. GPCRs are membrane bound receptors that contain seven hydrophobic transmembrane domains each containing between 25-40 amino acids. The transmembrane (TM) domains form α-helices of unequal length that can extend beyond the lipid bi-layer, and these form a counter clockwise barrel shape perpendicular to the membrane (Fig. 8). Of these domains TM II, IV and VII are the most hydrophobic as they contain only one hydrophilic residue, whereas the other domains contain a mixture of hydrophilic and neutral residues. GPCRs also have an extracellular N-terminus and a cytoplasmic C-terminus as well as 3 extracellular loops (EL) and 3 intracellular loops (IL). Each of these loops is between 10-40 amino acids in length except IL3 which can vary in length dramatically and contain up to 150 amino acids. GPCRs also have many conserved residues to ensure receptor activation and signalling. For example, Aspartate in TM II and Asparagine in TM III are conserved to keep TM II and VII in close proximity via hydrogen bonds enabling receptor activation (Ulloa-Aguirre et al., 1999).

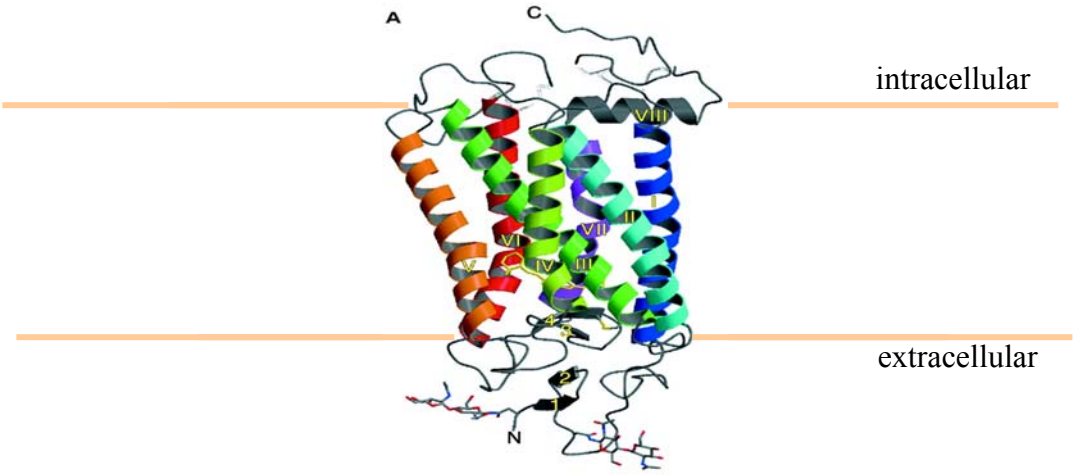

Figure 8. Structure of a Class A GPCR. Crystal structure of rhodopsin showing the counter clockwise orientation of the seven transmembrane domains. Diagram taken from Palczewski et al. *Crystal Structure of Rhodopsin: a G-protein coupled receptor* Science 2000. **289**: p739-745.

Gpr-54 is a typical class A GPCR in primary structure with an ORF of 1191 base pairs or 396 amino acids. The receptor gene contains 5 exons with 4 introns within TM II (800bp) and III (800bp) and ICL3 (180bp). Gpr-54 also contains many of the conserved residues found in this type of GPCR. Gpr-54 contains the conserved asparagine in TM I, the conserved aspartate in TM II mentioned above and conserved prolines in TM IV-VII. Gpr-54 also contains three consensus sequences for N-linked glycosylation at its N-terminus and a consensus sequence for PKC phosphorylation in IL3. Cysteines involved in the classical N-terminal disulphide bond are located in EL1 and EL2. There are also three possible palmitoylation cysteine sites in the C-terminus (Lee et al., 1999). The C-terminus of gpr-54 has also been shown to be rich in proline and arginine residues corresponding to four overlapping Src homology 3 (SH3) domain structures (Fig. 9). These SH3 domains have been shown to interact with the catalytic subunit of the serine/threonine (Ser/Thr) protein phosphatase, PP2A. The gpr-54/PP2A unit can then modulate phosphorylation of critical signalling intermediates. As PP2A has been shown to be a tumour suppressor this may be a mechanism for gpr-54 mediated inhibition of metastasis, as PP2A is known to up regulate phosphatase and tensin homology (PTEN) protein to down regulate the tumour promoter Akt, via dephosphorylation of PIP_2 (Evans et al., 2008).

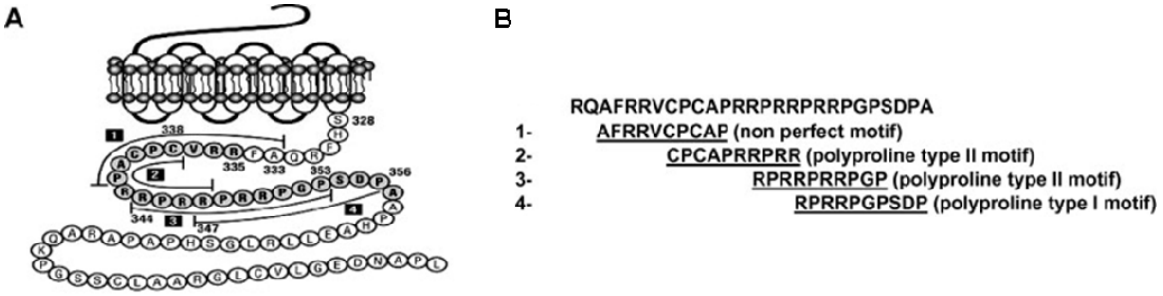

Figure 9. The C-terminal domain of gpr-54 contains a proline/arginine rich segment. (A). The C-terminus contains a region rich in Pro and Arg residues and few Ser and Thr residues. (B) The amino acid sequence of the Pro-Arg rich region contains four overlapping SH3 binding motifs. Diagram taken from Evans et al. *Physical association of GPR54 C-terminal with protein phosphatase PP2A.* Biochem Biophys Res Commun 2008. **377**(4): p1067-1071.

GPCRs signal by interacting with heterotrimeric g-proteins to cause conformational changes and an exchange of guanosine diphosphate (GDP) with guanosine triphosphate (GTP), allowing dissociation of Gα from the βγ subunit to activate intracellular signalling. Gpr-54 interacts with the $G\alpha_{q/11}$ subunit to activate specific signalling mechanisms (Kotani et al., 2001). Mutations in the receptor, have revealed that this interaction involves Leu^{148} of IL2 since dissociation of the g-protein subunits is disrupted by mutations at this residue (Wacker et al., 2008). IL2 of a gpr-54/β2-AR model forms hydrophobic interactions with the Gα subunit that stabilises the switch II region of the g-protein. This activates the subunit into a conformation that facilitates GDP-GTP exchange, allowing dissociation and maximising downstream signalling. Therefore, IL2 of gpr-54/β2-AR acts as a guanine nucleotide exchange factor (GEF). The Leu^{148} equivelant in β2-AR, Phe^{139}, has been shown to dock in close proximity to the GTPase domain of the $G\alpha_{q/11}$, where Pro^{138} interaction causes a change in IL2 to allow Phe^{139} to fit into the hydrophobic groove created by α2/β4 and α3/β5 loops of the g-protein (Wacker et al., 2008). Phe^{139} can then interact with Phe^{220}, Val^{223}, Trp^{263} and Phe^{264} of the switch II region to facilitate disociation of the Gα and βγ subunits (Fig. 10). This is now thought to be a mechanism common to all GPCR class A receptors.

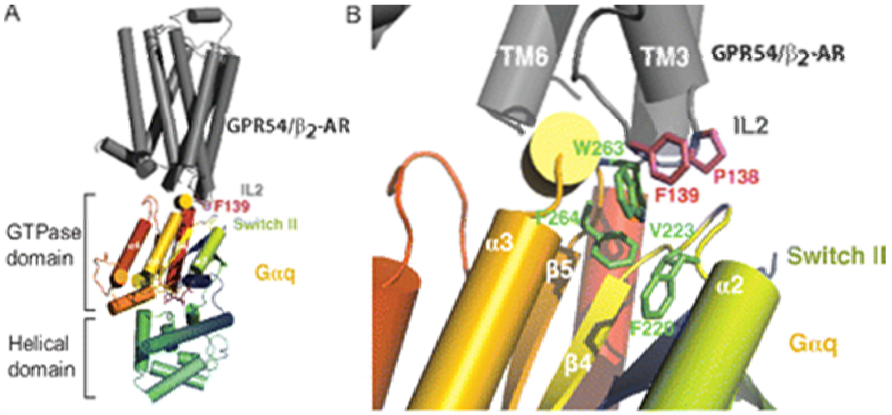

Figure 10. A model to suggest the involvement of IL2 in hydrophobic-hydrophobic interactions between GPCR and G-protein. (A) Docking analysis reveals that the IL2 of GPR54/β2-AR comes into close proximity to the GTPase domain of the activated $G\alpha_q$ subunit. (B) Pro^{138} of the β2-AR positions Phe^{139} so that a productive hydrophobic interaction face is formed with highly conserved Phe^{220}, Val^{223}, Trp^{263}, and Phe^{264} residues of $G\alpha_q$. Diagram taken from Wacker et al. *Disease-causing mutation in GPR54 reveals the importance of the second intracellular loop for Class A G-protein coupled receptor function.* J Biol Chem 2008. **283**(45): p31068-31078.

1.3. Kisspeptin is a key regulator of puberty and the HPG axis

1.3.1. Hypogonadotropic hypogonadism as a result of gpr-54 inactivating mutations.

KiSS-1 was first shown to play a role in the reproductive axis in 2003, when two groups discovered that mutations in gpr-54 led to idiopathic hypogonadotropic hypogonadism (iHH) (de Roux et al., 2003), which is a deficiency of gonadotropin secretion from the pituitary. De Roux's group found that in five affected siblings, from first cousin marriages, with normal GnRH receptor mRNA, a 155 nucleotide deletion in the gpr-54 receptor was the cause of their impaired puberty (de Roux et al., 2003). This deletion did not cause impaired migration of GnRH neurons from the olfactory bulb, so it could not be classified as Kallmann's syndrome, but was classified as iHH and is therefore due to disrupted stimulation of GnRH production (de Roux et al., 2003). Seminara et al found a separate gpr-54 mutation, L148S, in another family with 6 affected members also from first cousin marriages. This mutation caused impaired signalling of the gpr-54 receptor resulting in iHH. In the same study, a non-related male with iHH was also found to have gpr-54 mutations, R331X and X339R, causing elongation of the receptor sequence due to a disrupted stop codon (Seminara et al., 2003). Discovery of these mutations identified gpr-54 and kisspeptins as important regulators of puberty and the HPG axis. Since 2003, further mutations of the gpr-54 receptor have been found to cause iHH in humans. Semple et al. found C223R and R297L mutations in one male patient and these gave rise to impaired signalling, with C223R causing a more severe phenotype than R297L in the impairment of kisspeptin stimulation of calcium release *in vitro* (Semple et al., 2005). Another group reported that a base pair mutation 1001-1002insC caused iHH in association with cryptorchidism (Lanfranco et al., 2005). Then in 2007, 5 iHH patients were shown to have a L102P mutation in gpr-54 (Tenenbaum-Rakover et al., 2006). Gpr-54 mutations only account for about 2% of iHH cases (Pedersen-White et al., 2008) and all can be rescued by treating with GnRH or gonadotropins, which

induce puberty and normal reproductive function, allowing an affected individual to lead a normal reproductive life (Table 2).

Mutation	Phenotype	Receptor Impairment
155 nuc. deletion	iHH	truncation of receptor
L148S	iHH	impaired signalling
R331X & X339R	iHH	receptor elongation
C223R	iHH	impaired signalling
R297L	iHH	impaired signalling
L102P	iHH	impaired signalling
1001ins1002C	iHH & cryptorchidism	receptor elongation
R386P	Precocious puberty	prolonged signalling

Table 2. Activating and inactivating mutations of gpr-54. Table summarizing all inactivating and activating mutations of human gpr-54 reported to date. The table shows the mutation, phenotype and effect the mutation has on receptor sequence or signalling. References are shown in the text.

1.3.2. Precocious puberty as a result of gpr-54 and KiSS-1 mutations.

As well as inactivating mutations of gpr-54, a mutation, R386P (Table 2), apparently caused prolonged signalling of the receptor leading to precocious puberty in a girl of 8 years of age (Teles et al., 2008). A *KiSS-1* polymorphism prevalent in Chinese girls with precocious puberty, P110T, was also found to be statistically associated with the condition, although further evidence is needed to confirm that this substitution causes kisspeptin to constitutively activate the receptor (Luan et al., 2007).

1.3.3. KiSS-1 and gpr-54 knockout mice display characteristics of hypogonadotropic hypogonadism.

At the same time as gpr-54 mutations were being found to cause iHH in humans, a gpr-54 knockout mouse had been produced with similar findings to those from the patients described above. Gpr-54 -/- male mice had small testes, delayed puberty and reduced sexual behaviour and females had small ovaries and uteri, delayed vaginal opening, no maturation of follicles in the ovary and reduced sexual behaviour. Both sexes were infertile and had low gonadotropin levels, although low magnitude pulses could still be detected, suggesting that gpr-54 signalling is not required for basal GnRH or luteinising hormone (LH) levels in the mouse. Gpr-54 -/- mice are still responsive to exogenous GnRH, showing that there is an impaired secretion of GnRH in these animals (Lapatto et al., 2007; Seminara et al., 2003). The kisspeptin system, therefore appears to elevate GnRH pulse amplitude more than frequency (Seminara et al., 2003). These results confirm the human observations and identify kisspeptins and gpr-54 as major regulators of puberty.

More recently, KiSS-1 knockout mouse models have been developed that also display iHH characteristics, however the physiological effects are more variable than in the gpr-54 knockout. In one study, although infertile, only 50% of females had smaller uteri and ovaries with 50% being normal, however all had reductions in gonadotropins and sexual behaviour (Lapatto et al., 2007). In a different study, all *KiSS-1* -/- mice were infertile with delayed puberty. Female mice had delayed vaginal opening, small ovaries, thread like uteri, and no estrus cycle or follicle maturation due to decreased gonadotropin secretion. The male mice also had delayed pubertal maturation, small testes, microphallus, spermatogenic arrest and decreased gonadotropins and sex steroids (d'Anglemont de Tassigny et al., 2007). This shows the variability of the phenotype, as in one study the KiSS-1 knockout seems to be partially compensated for, but in the other the KiSS-1 knockout cannot be overcome by compensation. However, both sets of animals were responsive to exogenous kisspeptin, showing that both active kisspeptin

and gpr-54 are required for puberty to occur at the correct time. The less complete phenotype in the KiSS-1 knockout mice than gpr-54 knockout mice suggests that other RFamide peptides may be able to partially compensate for loss of kisspeptin via activating gpr-54 or their own receptors.

1.3.4. KiSS-1 mRNA and gpr-54 sensitivity in the hypothalamus increases with pubertal development.

The realisation of the pivotal role of the KiSS-1/gpr-54 system in reproduction stimulated further research in a quest to elucidate mechanistic details. *KiSS-1* and possibly *gpr-54* hypothalamic mRNA levels increase with pubertal maturation in mice (Han et al., 2005), rats (Sun et al., 2007) and primates (Keen et al., 2008; Shahab et al., 2005). In rodents, *KiSS-1* mRNA increases in the anteroventral periventricular nucleus (AVPV) but not in the arcuate nucleus (ARC) during puberty (Fig. 11 and Table 3). There is also an increase in the number of GnRH neurons that are depolarized by kisspeptin during puberty. In juvenile mice, only 27% of GnRH neurons depolarize with exogenous kisspeptin treatment, this increases to 45% in prepubertal mice and 90% in the adult. This increase in the number of GnRH neurons that are depolarized appears to be due to the increase in gpr-54 sensitivity at this time and the increased kisspeptin neuron apposition with GnRH neurons (Han et al., 2005), along with the increase in kisspeptin mRNA levels, which is shown to occur throughout puberty. In the mouse, kisspeptin neurons show close association with GnRH neurons in the preoptic area (POA) and median eminence (ME) and an increase with puberty (Fig.11). These neurons appear from postnatal day (P) 25 and reach adult like proportions of 50% co-localisation in the POA at around P61 (Clarkson and Herbison, 2006). As mentioned above, another explanation for the increase in depolarization is that GnRH neurons and gpr-54 gain sensitivity to kisspeptin during puberty in the mouse. Additionally, in prepubertal female rhesus monkeys, *KiSS-1* mRNA increases nocturnally and by mid-puberty *KiSS-1* and *gpr-54* mRNA increase 3-fold in the ARC. In males, *KiSS-1* mRNA increases with puberty but *gpr-54* mRNA does not, suggesting that increasing *KiSS-1* mRNA is a

controlling factor in males (Shahab et al., 2005). Also, in female rhesus monkeys, kisspeptin secretion increases during puberty in association with GnRH secretion and kisspeptin secretion in the ME becomes pulsatile with 60 minute intervals (Table 3). Of these pulses, 75% correlate with GnRH pulses (Keen et al., 2008). These pulses then continue into adulthood to regulate the HPG axis.

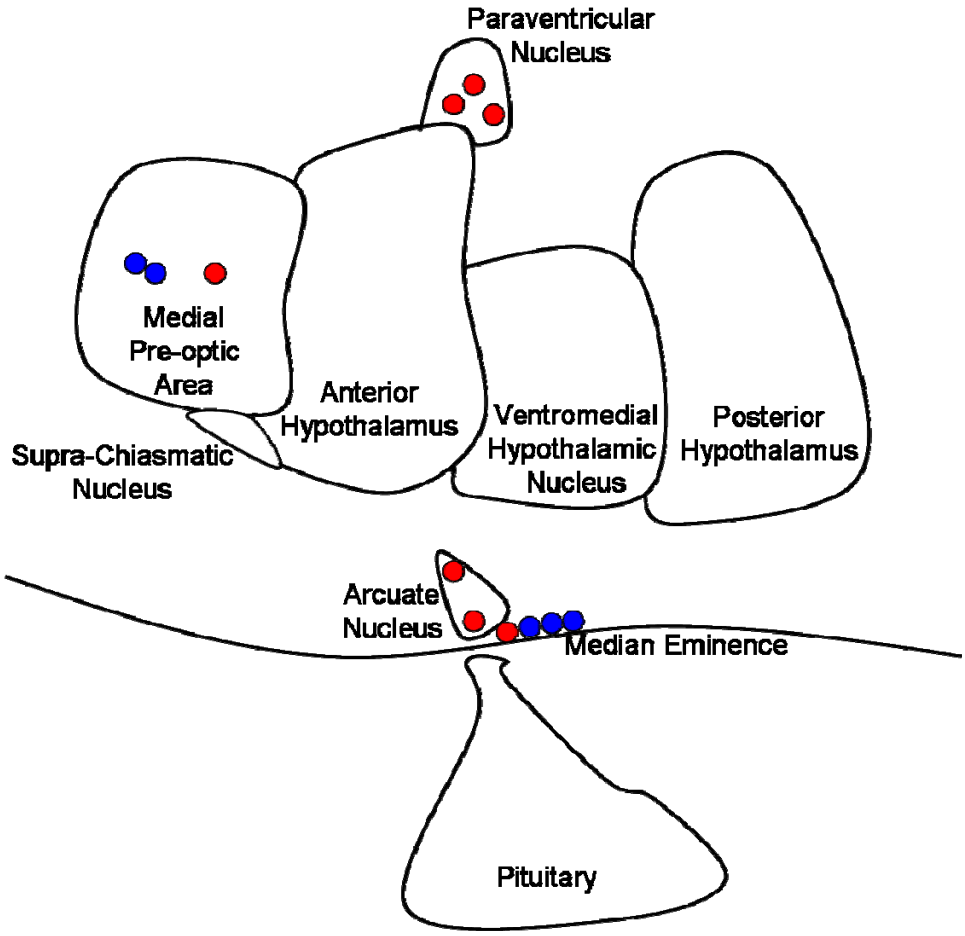

Figure 11. Schematic diagram of Hypothalamus and Pituitary. Diagram showing the layout of the hypothalamus in relation to the pituitary gland. Kisspeptin neurons (red) and GnRH neurons (blue) are marked in the appropriate areas.

In order to examine the physiological consequences of increased kisspeptin during puberty, studies were undertaken to determine the effect of repetitive administration of exogenous kisspeptin to juvenile female rats (Navarro et al., 2004b) and primates (Plant et al., 2006). This would reveal whether kisspeptin alone is sufficient to kick-start the onset of puberty. In both studies, repetitive kisspeptin administration was able to advance puberty, measured in the mouse as early vaginal opening and increased uterine weight and in both species gonadotropins were elevated to adult levels (Navarro et al., 2004b; Plant et al., 2006). In both cases the advance in puberty onset appears to be due to kisspeptin stimulation of GnRH secretion, as GnRH antagonists completely abolished the advancement of puberty.

These collective studies suggest that kisspeptin and gpr-54 are key regulators of puberty in mammals due to a programmed increase in *KiSS-1* mRNA and increased gpr-54 sensitivity to kisspeptin, possibly due to an increase in receptors at the cell surface. Activation of the kisspeptin system facilitates increased pulsatile release of GnRH, awakening the reproductive axis and bringing about pubertal maturation.

1.4. Kisspeptin and gpr-54 regulate the HPG axis in adults via modulation of GnRH, LH and FSH production

1.4.1. Acute kisspeptin stimulates secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) via GnRH.

As mentioned above, the expression of both ligand and receptor in the hypothalamus and pituitary respectively, and their crucial role in puberty suggests that kisspeptins may also be key regulators of the HPG axis in adults. In *gpr-54* -/- and *KiSS-1* -/- mice, LH levels were found to be significantly lower than in wild type (WT) mice even though GnRH receptor levels were normal and GnRH could elicit a robust LH stimulation. This indicated a role for kisspeptins in LH regulation in the adult. Kisspeptin-54 and

kisspeptin-10 rapidly increase plasma LH levels in adult mice (Gottsch et al., 2004), rats (Navarro et al., 2004a), sheep (Arreguin-Arevalo et al., 2006), cows (Kadokawa et al., 2008), primates (Plant et al., 2006) and humans (Dhillo et al., 2005; Dhillo et al., 2007) in a dose-dependant manner. Doses as low as 1fmol given centrally (intracerebroventricular (icv)) are effective (Gottsch et al., 2004), making kisspeptin the most potent stimulator of LH known to date. Low doses of 10pmol to 1nmol have also been shown to be active systemically (intraperitoneal (ip) and intravenous (iv)) in rodents (Navarro et al., 2005b; Thompson et al., 2004). The demonstration that kisspeptin stimulation of gonadotropins can be abolished by administering a GnRH receptor antagonist suggests that kisspeptin operates at the level of the hypothalamus to stimulate GnRH release rather than directly at the pituitary (Fig. 12) (Gottsch et al., 2004; Navarro et al., 2005b). Castellano et al. have attempted to delineate the mechanism behind kisspeptin activation of gonadotropins in the rat. Kisspeptin stimulation of LH could be blocked by PLC, calcium and MAPK inhibitors using hypothalamic explants pre-incubated for 60 min, however, no further evidence to support these pathways has been produced (Castellano et al., 2006b).

In gpr-54 and KiSS-1 knockout mice, follicle stimulating hormone (FSH) levels also are lower than in controls (Lapatto et al., 2007). Again, kisspeptin has been shown to stimulate release of FSH in adult rats (Navarro et al., 2005a), mice (Gottsch et al., 2004) and humans (Dhillo et al., 2005). However, in the rat a 100-fold higher dose of kisspeptin is required to stimulate FSH than for LH secretion, with an EC_{50} of 400nmol. Activation of FSH secretion also occurs via GnRH, as GnRH antagonist again blocks the kisspeptin stimulation (Navarro et al., 2005a). However, most studies have concentrated research on LH because FSH stimulation is of a much lower magnitude than that for LH. Further studies are required to fully elucidate kisspeptins effects on FSH production.

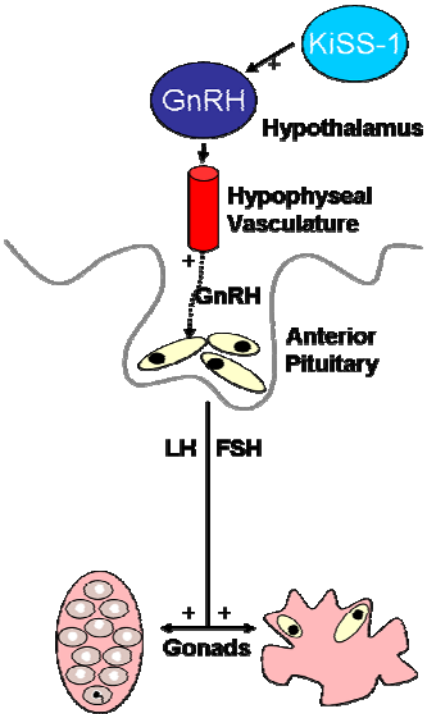

Figure 12. Kisspeptin stimulates GnRH release in the hypothalamus. Diagram showing kisspeptin stimulates GnRH release within the hypothalamus. This in turn causes secretion of gonadotropins from the pituitary into the circulation to act on the gonads.

1.4.2. Chronic kisspeptin administration disrupts secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH).

As described above, acute injection of kisspeptin stimulates LH release and repeated injections produce LH pulses (Tovar et al., 2006). Continuous iv injections of kisspeptin in rodents and primates over 4 days initially stimulate LH, but after 3-4 hours of stimulation this rise begins to fall, reaching control levels by 24 hours. This has been attributed to desensitization of the gpr-54 receptor, as GnRH injection is still able to increase LH secretion during the last 3 days of kisspeptin infusion in adult male mice and primates (d'Anglemont de Tassigny et al., 2008; Ramaswamy et al., 2007). Also, after the infusion is completed, acute kisspeptin administration cannot induce LH until the receptor recovers about 2 hours later. However, in female adult rats, the elevation of

LH lasted for 48 hours during a 7 day infusion and in peri-pubertal female rats, desensitization did not occur, suggesting a change in gpr-54 sensitivity during puberty or an increase in inputs to gpr-54 from kisspeptin neurons (Roa et al., 2008d). Nevertheless, it is evident that gpr-54 desensitizes with time, so chronic administration may be a useful tool therapeutically for inhibiting gonadotropins and sex steroids as currently accomplished by GnRH agonists in treating hormone-dependant diseases.

1.4.3. Kisspeptin neurons are located in the hypothalamus and directly contact GnRH neurons.

KiSS-1 mRNA and protein have been localised in the ARC and the AVPV of the rodent hypothalamus, with a small group of neurons also being identified in the periventricular nucleus (PeN) (Clarkson and Herbison, 2006; Gottsch et al., 2004; Kauffman et al., 2007a). However, in the ewe and in primates, kisspeptin neurons are not located within the AVPV (Franceschini et al., 2006; Rometo et al., 2007). The kisspeptin neurons project into the ME and POA regions within the hypothalamus, which also possess GnRH neurons (Franceschini et al., 2006; Pompolo et al., 2006). Double immunoflourescence has revealed that 85% of the GnRH neurons in the ME of rats contain gpr-54 on their cell surface and express increased levels of c-fos in response to kisspeptin-10 (Irwig et al., 2004; Matsui et al., 2004) . There is also a close association between 70-90% of GnRH and kisspeptin neurons in mice (Clarkson and Herbison, 2006), sheep (Pompolo et al., 2006) and rhesus monkey (Ramaswamy et al., 2008), suggesting that kisspeptin acts directly at the GnRH neurons to regulate the HPG axis. However, these associations differ in brain regions between different species. In the mouse, kisspeptin axons associate with GnRH cell bodies and dendrites in the POA (Clarkson and Herbison, 2006). In the sheep, co-localization is seen in both the ME and POA (Pompolo et al., 2006), and in the primate, associations occur in the ME but this time between kisspeptin and GnRH axons (Ramaswamy et al., 2008). However, it is yet to be determined if these differences are species related or simply due to differences in the primary and secondary fluorescent antibodies used in each study.

1.4.4. Kisspeptin and gpr-54 in the anterior pituitary.

The role of kisspeptin in the pituitary has been debated, with conflicting results obtained *in vitro* on primary pituitary cell cultures. Some groups have seen no effect of kisspeptin on rat anterior pituitary cells (Matsui et al., 2004), whereas another group showed increased Ca^{2+} in 10% of cells, along with stimulation of LH and growth hormone (GH) (Gutierrez-Pascual et al., 2007). However, the doses used in this study were extremely high compared to other studies and the effects may be due to activation of other RFamide receptors. LHβ-expressing cells within rat pituitary explants expressed *KiSS-1* mRNA; however *KiSS-1* mRNA was also detected in other pituitary cells, possibly somatotropes. *Gpr-54* mRNA was located in a subset of these LHβ expressing cells, suggesting the machinery is present for kisspeptin to have direct pituitary effects. Pituitary *KiSS-1* mRNA appears to be regulated by estrogen and gpr-54 appears to be regulated by GnRH, making it plausible for direct effects to occur in the rat (Richard et al., 2008). However, all of these studies have used highly sensitive PCR techniques that do not reveal whether expression of gpr-54 and kisspeptin is functionally significant. Moreover, pharmacological doses of kisspeptin were required to elicit effects on the pituitary.

In ovine anterior pituitary cells, *gpr-54* mRNA is present in gonadotropes, somatotropes and lactotropes. Administering kisspeptin to these cells when taken during the follicular phase causes an 80% increase in LH release. However, anterior pituitary cells taken from all other stages of the ovine cycle did not respond to kisspeptin administration. Also, in ovariectomised (OVX) ewes which were hypothalamus-pituitary disconnected (hence ablating effects of GnRH on the pituitary); no stimulation of LH by kisspeptin was seen at any time during the cycle (Smith et al., 2008). Furthermore, although kisspeptin is present at low levels in the hypophysial blood, this was not affected by estrogen, even at LH surge levels (Smith et al., 2008). Thus *in vivo* findings in contrast to *in vitro* findings indicate that direct pituitary effects of kisspeptin are not important

for LH release. Overall, there is no convincing data demonstrating that direct effects of kisspeptin at the level of the pituitary are important for regulation of the HPG axis.

1.5. Kisspeptin and gpr-54 mediate steroid feedback on the HPG axis

1.5.1. Kisspeptin and gpr-54 mediate negative steroid feedback within the HPG axis.

It has been known for many years that the HPG axis is under the control of steroid hormone feedback from the gonads. Steroid hormone levels fluctuate across the cycle in females (Almond and Dial, 1990; Gill et al., 2002; Karsch and Evans, 1996; Moenter et al., 2003). However, the mediator of steroid hormone feedback has remained elusive for many years, as GnRH neurons only possess estrogen receptor β (ERβ) which does not play a role in feedback (Herbison and Pape, 2001). Therefore, neurons upstream of the GnRH neuron, which possess estrogen receptor α (ERα), progesterone receptor (PR) and androgen receptor (AR) have been sought as possible mediators of steroid effects on GnRH release.

Kisspeptin neurons express ERα, PR and AR and therefore have the potential to relay feedback effects onto the GnRH neuron. Regulation of KiSS-1 expression is likely be a mediator of negative feedback in mouse (Smith et al., 2005a; Smith et al., 2005b), rat (Adachi et al., 2007), ewe (Franceschini et al., 2006; Goodman et al., 2007; Smith et al., 2006b) and human (Rance, 2008). Evidence now suggests that reduced activity of kisspeptin neurons in the ARC of rodents, primates and sheep is responsible for translating estrogen negative feedback to GnRH neurons. OVX female and castrated male mice (Smith et al., 2005a; Smith et al., 2005b), sheep (Pompolo et al., 2006; Smith et al., 2006b) and rhesus monkeys (Rometo et al., 2007; Shibata et al., 2007) have an increased level of *KiSS-1* mRNA in the neurons compared to controls (Table 3). Also, if estrogen replacement is given to OVX female or testosterone is given to castrated male

mice, sheep and rats, then *KiSS-1* mRNA levels are reduced to control levels (Pompolo et al., 2006; Rometo et al., 2007; Shibata et al., 2007; Smith et al., 2006b; Smith et al., 2006c; Smith et al., 2005b). This suggests that steroids are negatively regulating *KiSS-1* mRNA in the ARC, hence reducing stimulation of GnRH neurons. *KiSS-1* mRNA regulation by steroids is specifically mediated via the ERα isoform as PPT, a specific inhibitor for this ER isoform blocks the reduction of neuronal *KiSS-1* mRNA in the ARC, but DPN an ERβ specific inhibitor does not affect these mRNA levels (Bateman and Patisaul, 2008). Also ERα -/- mice do not have any steroid negative feedback on KiSS-1 mRNA levels in the hypothalamus (Smith et al., 2005a).

This negative regulation of kisspeptin by steroids was also revealed by analyses of *KiSS-1* mRNA regulation across the rat ovarian cycle in the ARC (Table 3), where levels increased at diestrus when estrogen levels are low and decreased at proestrus when estrogen levels are elevated (Adachi et al., 2007). Further evidence for negative feedback is derived from females at the time of menopause, when estrogen is low due to reduced follicle numbers in humans and rhesus monkeys. At this time, a rise in *KiSS-1* mRNA and in turn LH is seen accompanied by cellular hypertrophy, similar to the rise in OVX female rhesus monkey (Kim et al., 2008; Rance, 2008; Rometo et al., 2007), which is thought to be due to the lack of negative feedback from the follicles.

However, kisspeptin cannot be the only factor effecting GnRH secretion, since in the gpr-54 knockout mouse, there are still basal pulses of LH (Colledge, 2009; Seminara et al., 2003). It has been shown that KiSS-1 co-localises with two other groups of ERα, PR and AR positive peptidergic neurons; the dynorphin A neuron and the neurokinin B neuron (Goodman et al., 2007). Recently, a mutation in the neurokinin B receptor in man has been shown to affect the HPG axis and reproduction. Therefore, it is possible that the different types of neuron work in concert to regulate negative feedback and the HPG axis in the ARC (Topaloglu et al., 2009). However, this may not be the case in mice, where neurokinin B knockouts do not display a reproductive phenotype (Nordquist et al., 2008; Siuciak et al., 2007).

1.5.2. Kisspeptin and gpr-54 mediate positive steroid feedback via an anatomically distinct neuronal pathway.

As well as eliciting negative feedback, estrogen also induces positive feedback on the HPG axis at the time of the pre-ovulatory LH surge in females. The LH surge occurs when estrogen reaches a threshold level synchronising GnRH neuron secretory activity to stimulate the LH surge for ovulation, followed by a secondary FSH surge on the morning of estrus in rats (Roa et al., 2008a). Again, due to the lack of ERα in GnRH neurons, estrogen appears to act upstream of these neurons. Kisspeptin neurons are perfectly placed to mediate this response. In mice and rats, positive feedback is regulated by a separate group of kisspeptin neurons in the AVPV, which are associated with the LH surge (Adachi et al., 2007; Smith et al., 2005a; Smith et al., 2006d). This association can be demonstrated at the level of the neuron, where *KiSS-1* mRNA is decreased in the AVPV of OVX female rats and its levels increase with estrogen replacement (Table 3) (Smith et al., 2005a). Positive feedback on *KiSS-1* mRNA in the AVPV can also be shown in intact female rats, where AVPV *KiSS-1* mRNA increases on the afternoon of proestrus (Table 3) and expression of c-fos increases in kisspeptin neurons just before the LH surge when estrogen is at its highest levels (Adachi et al., 2007). The kisspeptin neurons in the AVPV may project to the POA as kisspeptin blockade in the POA, using neutralising antibodies, inhibits the LH surge, proving the importance of kisspeptin neurons in positive feedback (Adachi et al., 2007). As with negative feedback, positive feedback is regulated through ERα, as blockade of this receptor completely inhibits the gonadotropin surge and ovulation in mice and rats (Adachi et al., 2007; Kinoshita et al., 2005; Roa et al., 2008c).

In contrast to mice and rats, in sheep and primates kisspeptin neurons are only localised to the ARC and PeN and not the AVPV. Here, the ARC appears to be responsible for both negative and positive feedback (Estrada et al., 2006; Pompolo et al., 2006; Smith et al., 2007). In adult ewe neurons, *KiSS-1* mRNA in the caudal ARC rises during the follicular phase and in the rostal ARC at estrus when the LH surge occurs (Estrada et al.,

2006). This suggests the ARC is responsible for modulating the positive steroid feedback in the ewe. In the intact ewe, during the estrus cycle, kisspeptin can synchronise LH surges and during anoestrus, administration of kisspeptin can cause ovulation to occur, suggesting that when kisspeptin levels are high enough they can stimulate the LH surge and they are therefore probably involved in relaying positive feedback to GnRH neurons (Caraty et al., 2007).

1.5.3. Steroids are responsible for sexual dimorphism of KiSS-1 expression in neurons in the rat AVPV.

In the AVPV of rats, females have 12-fold higher *KiSS-1* mRNA expression than males, probably in order to produce a synchronised LH surge. This elevation appears to be due to steroid exposure during neonatal life. If female rats are exposed to testosterone neonatally, they develop male levels of kisspeptin in the AVPV and conversely male gpr-54 knockout mice develop female levels, due to low testosterone levels. However, kisspeptin levels in the rat ARC are not affected (Kauffman et al., 2007a; Kauffman et al., 2007b) . This suggests that testosterone in neonatal life, in both males and females, is important in developmental differentiation of kisspeptin neurons in the AVPV.

Overall, it is apparent that kisspeptin neurons are important mediators of steroid feedback in many species, to regulate LH release throughout the oestrous cycle and to synchronise release at the time of the GnRH/LH surge (Fig. 13). Evidently, steroids are important regulators of kisspeptin neurons and play an important role in the regulation of the HPG axis in developmental, prepubertal and adult life.

Species	Action	Study	KiSS-1 mRNA			KiSS-1 immunostaining			Gpr-54 mRNA			Reference
			AVPV	ARC	ME	AVPV	ARC	ME	AVPV	ARC	ME	
Mouse	Puberty	Puberty onset	↑	no change							no change	Han et al, 2005
	Steriod feedback	OVX/Castration	↓	↑							↓	Smith et al, 2007a
		+ Estrogen	↑	↓								Smith et al, 2007b
	Metabolic regulation	Ob/ob mouse		↓							↓	Smith et al, 2006a
		+ Leptin		↑								Luque et al, 2007
		Starvation	↓	↓							↓	Luque et al, 2007
Rat	Puberty	Puberty onset					↓					Sun et al, 2007
	Postive feedback	OVX (proestrus)	↑									Adachi et al, 2007
	Negative feedback	OVX (estrus)		↑								Adachi et al, 2007
		OVX (diestrus)		↓								
Hamster	Photoperiod	Siberian (Long Days)	↑	↓		↑	↓					Mason et al, 2007
		Siberian (Short Days)	↓	↑		↓	↑					Greives et al, 2006
		Syrian (Long Days)	n/a	↑								
		Syrian (Short Days)	n/a	↓								Revel et al, 2006
		Syrian (Pineal Ablation)	n/a	↑								
Sheep	Negative feedback	OVX/Castration	n/a	↑								Pompolo et al, 2006
		+ Estrogen	n/a	↓								Smith et al, 2006b
	Postive feedback	Follicular stage (Caudal)	n/a	↑								Estrada et al, 2006
		LH surge (Rostal)	n/a	↑								
	Photoperiod	Long Days	n/a	↓								Smith et al, 2006b
		Short Days	n/a	↑								
Primate	Puberty	Puberty onset	n/a	↑							↑	Shahab st al, 2005
	Negative feedback	OVX/Castration	n/a	↑								Rometo et al, 2007
		+ Estrogen	n/a	↓								Shibata et al , 2007
Human	Negative feedback	Post-Menapause				n/a	↑					Rance, 2008

Table 3. Regulation of *KiSS-1* and *gpr-54* within the hypothalamus. Table showing the effects on *KiSS-1* and *gpr-54* mRNA within the hypothalamus in response to different stimuli such as steroid hormones and puberty.

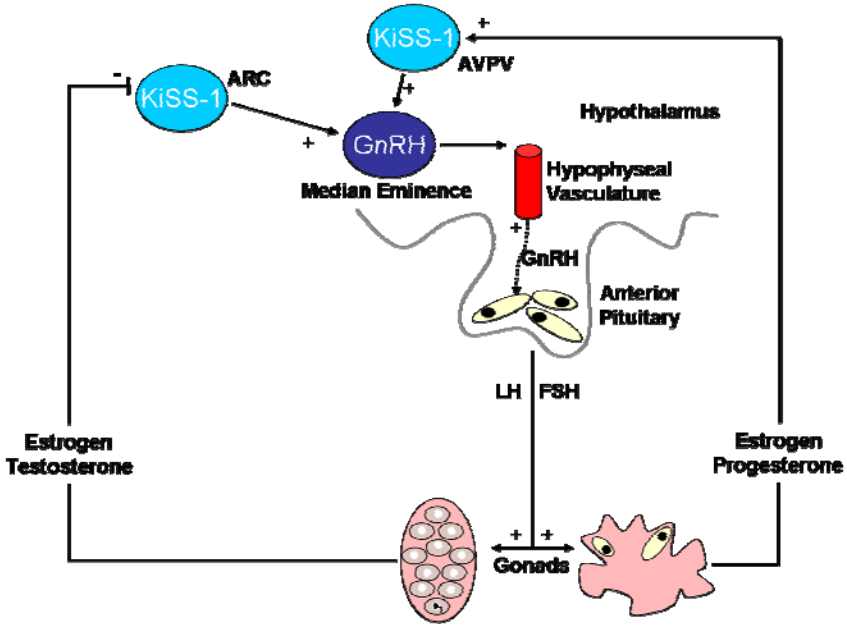

Figure 13. Kisspeptin mediates positive and negative steroid feedback. Diagram showing positive and negative feedback inputs into the HPG axis through KiSS-1 neurons in the AVPV and ARC within the hypothalamus of rodents, which project to co-localise with GnRH neurons in the preoptic area and median eminence. In sheep and primates, the ARC but not the AVPV is involved in both negative and positive feedback.

1.6. KiSS-1 neurons are influenced by environmental and metabolic factors

1.6.1. Kisspeptin is regulated by photoperiod in seasonal breeders.

Environmental factors such as photoperiod play a regulatory role in the HPG axis via regulation of GnRH production (Porkka-Heiskanen et al., 1997). In view of its major role in the regulation of the GnRH neuron, kisspeptin neurons were hypothesised to relay photoperiod effects on GnRH neurons in seasonally breeding species. In seasonal breeders such as sheep, cattle and hamsters, activation of the reproductive axis is controlled by melatonin produced from the pineal gland, which is secreted at night only and therefore relays day-length to the body (Bittman et al., 1985). Three animal models have been employed to look at these effects, the Siberian and Syrian hamsters (long day breeders), and sheep (short day breeders; see Table 3).

Female Siberian hamsters are sexually active during long days (LD), when melatonin secretion is reduced. During this time *KiSS-1* levels in the ARC are decreased, while AVPV *KiSS-1* levels are increased, to give optimum conditions for conception (Mason et al., 2007). However, during short days (SD) when female Siberian hamsters are in anoestrus the opposite occurs, with high *KiSS-1* levels in the ARC and low levels in the AVPV (Table 3) (Greives et al., 2006; Mason et al., 2007). In the Syrian Hamster, also a long day breeder, no staining was found in the AVPV, but an increase in *KiSS-1* staining was seen in the ARC during LD and a decrease was seen during SD. This response to seasonal breeding appears to be a result of melatonin production as pineal gland ablation prevents the reduction in ARC *KiSS-1* during SD (Revel et al., 2006). Thus melatonin is important for *KiSS-1* regulation in both Siberian and Syrian hamsters but evidence of functional melatonin receptors on kisspeptin neuronal cells has not been demonstrated so far.

In the ewe, which is a short day breeder and so responds to a long melatonin secretion period, *KiSS-1* mRNA in the ARC is increased during SD when oestrus occurs, and ARC *KiSS-1* expression is decreased during LD for anoestrus (Table 3) (Smith et al., 2006b). This allows activation of the LH surge during oestrus but inhibition during anoestrus (a mechanism which has evolved to co-ordinate reproduction with seasonal availability of food). However, seasonal endocrine physiology can be overcome with exogenous kisspeptin, which when given continuously for 30 hours can cause ovulation in 80% of ewes during anoestrus (Caraty et al., 2007). This shows that kisspeptin is a potent regulator of the HPG axis, even when the system is dampened.

Therefore, kisspeptin neurons represent a conserved mechanism for relaying photoperiodic cues in seasonal breeding animals (Fig. 14). The neuronal networks and melatonin actions are yet to be delineated.

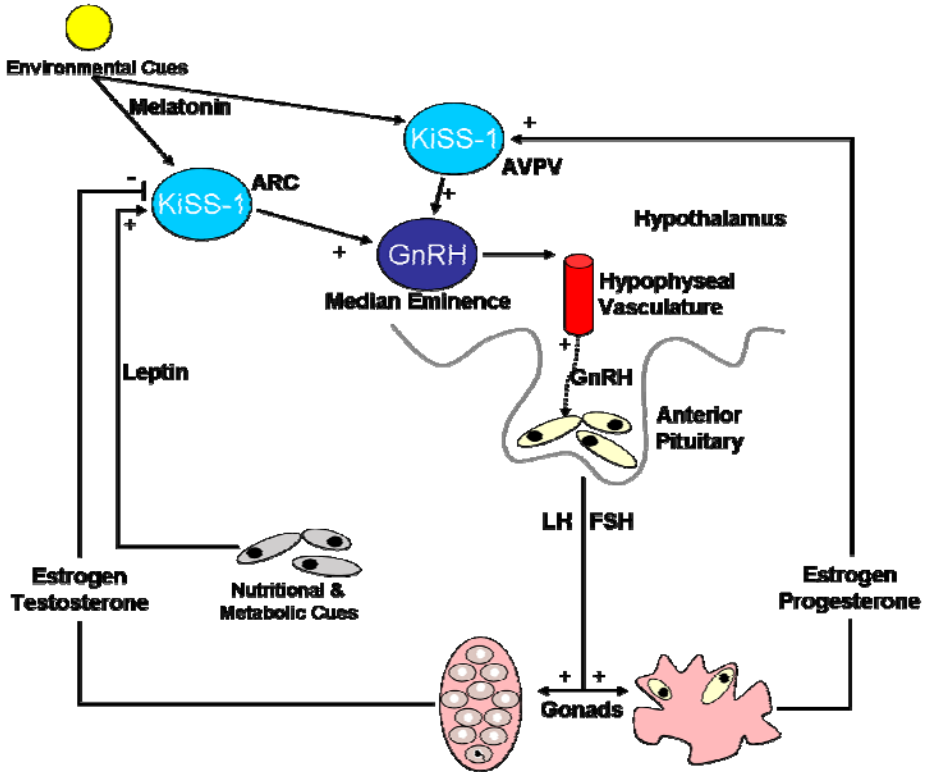

Figure 14. Kisspeptin mediates environmental and metabolic signals to GnRH. Diagram showing that as well as mediating steroid feedback, KiSS-1 neurons also integrate signals from the environment via melatonin and from metabolism via leptin and then relay these signals to the GnRH neuron.

1.6.2. Kisspeptin is regulated via Leptin signalling.

Metabolic factors, such as body mass index (BMI) have profound effects on reproduction. This is manifestly evident in female dancers and athletes when body weight declines to low levels during intense training, resulting in amenorrhea (Shade, 1983). Leptin, an adipose-derived hormone is known to affect metabolism, food intake and reproduction (Bluher and Mantzoros, 2007) and leptin increase is proposed to regulate the onset of puberty (Bluher and Mantzoros, 2007). Around 40% of kisspeptin neurons in the mouse possess leptin receptors (Smith et al., 2006a); this implicates kisspeptin neurons as mediators of metabolic control of the HPG axis and puberty.

In order to examine the effect of leptin on KiSS-1, two models, the ob/ob mouse, which lacks leptin due to a spontaneous single base pair mutation, and states of under-nutrition have been studied. In the ob/ob mouse, *KiSS-1* expression in the ARC is reduced compared to controls (Table 3) and this is partially rescued by leptin replacement (Smith et al., 2006a). The same is also true for *gpr-54* mRNA, indicating that leptin can regulate *KiSS-1* and *gpr-54* mRNA (Luque et al., 2007). In adult mice subjected to short term fasting, there is a rapid decline in both *KiSS-1* and *gpr-54* mRNA (Table 3) at around 12-24 hours followed by a decrease in GnRH at 48 hours (Luque et al., 2007). In fasting prepubertal rats, *KiSS-1* mRNA is still decreased but *gpr-54* mRNA is increased at this stage (Castellano et al., 2005). However, opposite gpr-54 responses are seen in underfed animals subjected to continuous kisspeptin infusion. In the presence of continuous kisspeptin infusion, underfed adult female rats, have a prolonged LH response to kisspeptin, which lasts 5 days, compared to 2 days in control rats and in underfed female peri-pubertal rats, LH responds for the entire seven days with no sign of receptor desensitization (Roa et al., 2008d). These results suggest that leptin positively regulates *gpr-54* mRNA in pubertal and adult life (Figure 13), as these responses were mimicked by leptin administration. Induction of *gpr-54* mRNA appears to be more sensitive to leptin during puberty than in adults. The above evidence places kisspeptin and gpr-54 as major mediators of metabolic signals to GnRH and the HPG axis.

1.7. Kisspeptin and gpr-54 may have direct actions at peripheral sites

1.7.1. Kisspeptin expression and effects in the ovary.

Expression of KiSS-1 and gpr-54 has been detected in peripheral tissues in addition to the hypothalamus and other brain regions. Expression has been noted within the testis and the ovary. No functional effect of kisspeptin has been found in the testis, but regulation of *KiSS-1* mRNA around the time of ovulation has been noted in the ovary. In the rat ovary, *KiSS-1* and *gpr-54* mRNA expression is found in the ovarian surface

epithelium (OSE) and interstitial glands of the stroma at all stages of the cycle. Whereas *KiSS-1* and *gpr-54* expression in the follicle was stage dependant, with staining in the theca layer of growing and preovulatory follicles from oestrus to early proestrus, which then switched to the granulosa cell layer of preovulatory follicles in late proestrus. After ovulation, expression returned to the theca-lutein cells of the corpus luteum and decreased as the corpus luteum regressed. Also expression levels fluctuated with the oestrus cycle with expression increasing on the afternoon of proestrus just preceding the LH surge and ovulation and then sharply decreasing thereafter. The increase in *KiSS-1* mRNA seems to be directly associated with the increase in LH at the time of the surge. If the LH surge is absent then this rise does not occur. Administration of human chorionic gonadotropin (hCG), to rats, increased *KiSS-1* mRNA in the ovary, suggesting the ovarian rise in *KiSS-1* mRNA is regulated by LH. In prepubertal female rats, *KiSS-1* expression in the ovary is very low, due to low LH levels, but this can again be overcome by hCG administration (Castellano et al., 2006a). Therefore, kisspeptin and gpr-54 show spatial and temporal expression changes in the ovary during the oestrus cycle, which suggests a functional role.

KiSS-1 and *gpr-54* expression has also been reported in the human and marmoset ovary. In both species, *KiSS-1* mRNA can be seen in the follicle, corpus luteum, interstitial glands and the OSE, whereas gpr-54 mRNA was only found in the thecal and luteal cells of the follicle. The mechanism of *KiSS-1* regulation in the ovary was also investigated in this study. *KiSS-1* mRNA but not *gpr-54*, *cyclooxygenase (COX)-2* or *progesterone* mRNA was dramatically inhibited with the COX inhibitor, indomethicin. The decrease is due to inhibition of COX-2 as a specific inhibitor of this, NS398 but not a COX-1 inhibitor, SC506, caused the same phenomenon. The decrease could also be rescued by the prostaglandin, PGE_2 (Gaytan et al., 2009). This places the prostaglandin pathway as a novel regulator of *KiSS-1* expression in the ovary.

1.7.2. Kisspeptin and the placenta

One of the largest peripheral sources of kisspeptin in the human is the placenta. In the human placenta, *KiSS-1* mRNA is located within the syncytiotrophoblast cells (Bilban et al., 2004; Horikoshi et al., 2003) and *gpr-54* mRNA is located in the syncitio-, villous and extravillious trophoblast cells (Fig. 15), suggesting possible paracrine actions (Bilban et al., 2004). *KiSS-1* mRNA and protein are increased with pregnancy in humans, and in the placenta appears to be highest in the first trimester (Bilban et al., 2004). However, in the maternal plasma KiSS-1 protein dramatically increases throughout pregnancy. In non-pregnant plasma the kisspeptin concentration is around 1.3fmol/ml this increases to ~1230fmol/ml in the first trimester and rises to ~9590fmol/ml at term. After delivery kisspeptin levels return to normal within 5 days suggesting the source of kisspeptin is the placenta (Horikoshi et al., 2003), this is in contrast to the decreasing levels within the placenta itself. *Gpr-54* mRNA and protein is also found in the placenta and levels are shown to be highest in the first trimester with a steady decline until term (Bilban et al., 2004). This suggests a role in implantation and placentation. Similar results are also seen in the mouse with *KiSS-1* and *gpr-54* expression only found in the cytotrophoblasts. Again, *gpr-54* is highest in the first trimester but *KiSS-1* expression appears constant throughout the pregnancy, suggesting possible species differences (Janneau et al., 2002). *KiSS-1* appears to be a conserved regulator of placentation as it can also be located in the trophoblast giant cells within the rat placenta, were again *KiSS-1* and *gpr-5*4 is highest early in placentation at embryonic day (E) 12.5 but has been completely lost by E18.5 (Terao et al., 2004).

The role of the kisspeptin system in the placenta is thought to be the regulation of trophoblast cell invasion. Trophoblast invasion of the uterine deciduum is important to allow remodelling of the maternal arteries, to provide sufficient blood flow to the developing foetus. This requires a balance of inhibitory and stimulatory signals to the invading trophoblast cell population. Kisspeptin-10, the form found in the placenta (Bilban et al., 2004) has been shown to inhibit migration of primary trophoblast explants

and primary cell cultures and this is related to a decrease in MMP expression (Bilban et al., 2004). Kisspeptin and gpr-54 may also be important in the pathophysiology of the placenta. In pre-eclampsia, where trophoblast invasion is shallow, *KiSS-1* mRNA and protein are elevated and these increase with disease severity. This has been correlated with a decrease in the expression of MMP-9 and decreased birth weight (Qiao et al., 2005). *KiSS-1* expression is also significantly higher in the placenta during pre-term labour but circulating levels do not change (Torricelli et al., 2008). This suggests that during pregnancy, placental kisspeptin is very important and that its primary role is to down regulate MMPs to inhibit trophoblast invasion during placentation.

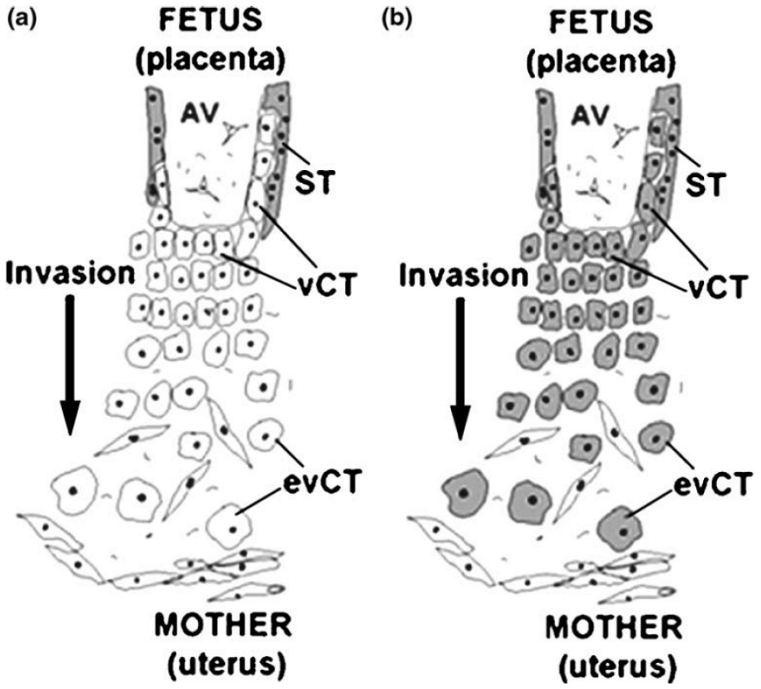

Figure 15. KiSS-1 and gpr-54 expression in the placenta. (A) KiSS-1 expression within the syncytiotrophoblasts (ST) of the invading placenta (shaded cells). (B) Gpr-54 expression within the syncytiotrophoblasts (ST), villous (vCT) and extravillious (evCT) cytotrophoblasts of the invading placenta (shaded cells). Diagram taken from Hiden et al., *Kisspeptins and the placenta: Regulation of trophoblast invasion* Rev Endocr Metab Disord, 2007. **8**(1): p 31-39.

1.7.3. Putative role of kisspeptin in cancer metastasis

Kisspeptin was first discovered as an anti-metastatic factor in cancer tissues. However, since then data has been varied and at times contradictory. In many cancer tissues such as clear cell ovarian cancer (Prentice et al., 2007), epithelial ovarian cancer (Hata et al., 2007), pancreatic cancer (Katagiri et al., 2009; Masui et al., 2004; Nagai et al., 2009) and melanomas (Nash et al., 2007), kisspeptin and gpr-54 expression have been positively correlated with increased patient survival. However in other cancers such as estrogen positive breast cancer (Marot et al., 2007), transition cell carcinoma of the bladder (Nicolle et al., 2006) and hepatocellular cancer (Schmid et al., 2007), kisspeptin and gpr-54 have been correlated with decreased survival. Yet, in estrogen positive breast cancers, kisspeptin is also associated with decreased invasion (Marot et al., 2007). Therefore, it would appear that even though kisspeptin is not always associated with a positive outcome, it does appear to inhibit cancer cell invasion.

Different mechanisms of function have been hypothesised for this inhibition of metastasis. Some publications have shown an increase in ERK1/2 phosphorylation and a decrease in MMP-2 as described in the placenta (Masui et al., 2004; Ringel et al., 2002; Yoshioka et al., 2008). Other suggested mechanisms include antagonism of stromal cell-derived factor-1 (SDF-1) signalling to inhibit the pro-metastatic properties of its chemokine receptor CXCR4. This may involve the up regulation of modulatory calcineurin-interactin protein-1 (MCIP-1), a chemokine capable of inhibiting the calcinuerin signalling pathway (Navenot et al., 2005; Stathatos et al., 2005). Another hypothesis is that kisspeptin is regulated by Specificity Protein 1 (SP1) and its co-activator DRIP130 which is located on chromosome region 6q16.3q23. When Loss of hetrozygosity (LOH) occurs at this region KiSS-1 gene expression is frequently lost from tumours and allows metastasis to occur. This can be rescued by SP1 and DRIP130 transcription factor which can lead to inhibition of invasion and migration (Mitchell et al., 2007; Shirasaki et al., 2001). However, further research is needed to delineate the signalling pathways further and to evaluate an association with cancer prognosis.

1.7.4. Kisspeptin and gpr-54 are expressed in non-reproductive tissues

The kisspeptin/gpr-54 system has been shown to have effects in other regions of the brain and periphery not connected with reproduction. In the brain, *KiSS-1* and *gpr-54* mRNA are located in the hippocampal dentate gyrus, where kisspeptin-10 can up regulate the excitatory post-synaptic current (EPSC) of the dentate granule cells by increasing the amplitude but not frequency of the current. This increase in synaptic transmission is via a MAPK pathway, which may be regulated via camodulin-dependant kinase (CaMK) and tyrosine kinase mechanisms (Arai et al., 2005). *KiSS-1* mRNA is up regulated in kainite-induced seizures in the hippocampus of rats via a α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) dependant mechanism. Kisspeptin can also increase brain-derived neurotrophic factor (BDNF) levels in the hippocampus, a factor that is critical for hippocampus function and correlates with the severity of seizures in this region (Arai and Orwig, 2008).

In peripheral tissues, kisspeptin has a putative role as a vasoconstrictor in aortic smooth muscle. In addition *KiSS-1* and *gpr-54* mRNA have been localised to the vascular endothelial cells and atherosclerotic plaques of the coronary artery. However, further work is needed to discover the relevance of this vasoconstriction and if this system is active *in vivo* (Mead et al., 2006). In the adrenal gland, *gpr-54* mRNA has been localised to the neocortex of human adrenals, with a 50% increase in foetal compared to adult adrenal glands. In this gland, kisspeptin-54 can stimulate secretion of aldosterone into the kidney vasculature to regulate blood fluid levels and blood pressure (Nakamura et al., 2007). In the pancreas, *KiSS-1* and *gpr-54* mRNA have been found in human and mouse islet cells of the endocrine pancreas, where kisspeptin can augment release of glucose-induced insulin in a high-glucose environment (20mmol/l) in humans and rats. This increase in insulin acts via receptors in the periphery as iv but not icv injection stimulates this increase and this has again been shown to involve the ERK1/2 pathway (Bowe et al., 2009; Hauge-Evans et al., 2006). This set of studies indicates that kisspeptin may regulate a wide range of effects in the body.

1.8. Signalling Pathways activated by gpr-54

To date a limited number of signalling pathways have been evaluated for involvement in the kisspeptin/gpr-54 pathway. These evaluations have predominantly been in relation to two areas of kisspeptin action: GnRH secretion from the GnRH neuron and kisspeptin actions in cancer metastasis. This section will focus on the pathways activated in these two settings. As mentioned in section 1.1.2, kisspeptin signalling mechanisms were first investigated in CHO cells stably expressing gpr-54, where kisspeptin stimulation, through $G_{q/11,}$ activated PLC to hydrolyse PIP_2 to IP_3, which increased intracellular calcium release and generated DAG, which activated PKC and this in turn activated the MAPK pathway.

1.8.1. Kisspeptin signalling in GnRH neurons

KiSS-1 neurons directly contact GnRH neurons expressing gpr-54 to cause secretion of GnRH into the hypophysial portal blood to act on the pituitary. The signalling mechanisms of this secretion have recently been delineated. Using GnRH neurons from GFP-transgenic mice, it was shown that kisspeptin causes depolarisation of GnRH neurons in a dose-dependant manner. This occurs via activation of sodium-dependant, non-selective cationic channels, possibly TRPC-like channels as the depolarisation is blocked by 2-APB an inhibitor of TRPC channels. This activation is also accompanied by inhibition of inwardly rectifying (Kir) potassium channels (Liu et al., 2008; Zhang et al., 2008). This inhibition may be due to a blockade of $GABA_B$ receptors, as kisspeptin has been shown to inhibit $GABA_B$ and the subsequent Kir potassium channel activation (Zhang et al., 2009). This mechanism of cationic channel activation and potassium channel inhibition is dependant on PLC and the IP_3 receptor, and therefore influences both plasma membrane and endoplasmic reticulum-driven calcium release (Fig. 16). Plasma membrane tetradotoxin-sensitive channels have also been implicated in GnRH neuronal depolarisation (Constantin et al., 2008).

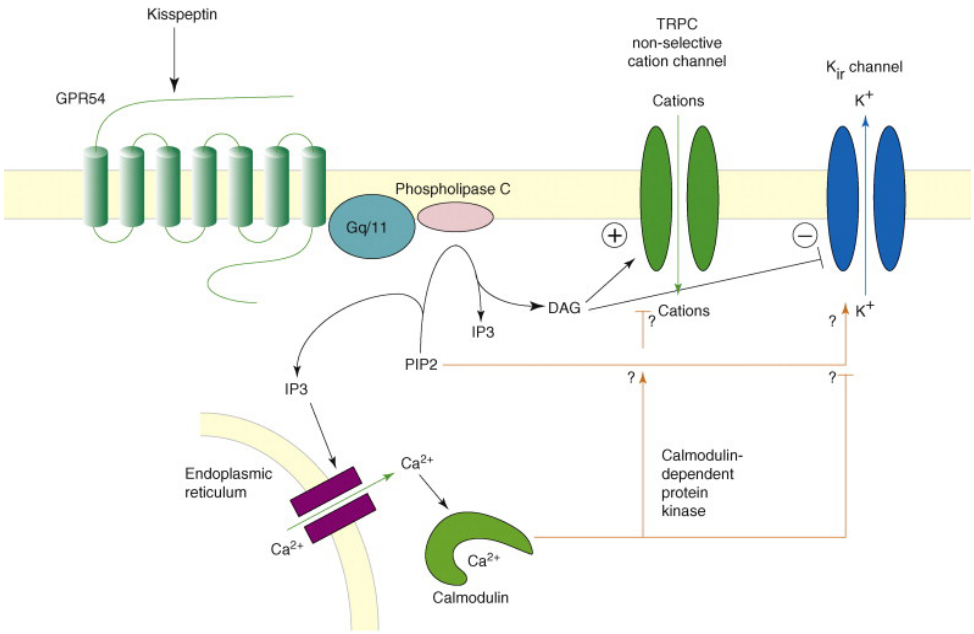

Figure 16. Kisspeptin signalling in the GnRH neuron. Schematic diagram of the signalling pathways and ion channels operated by kisspeptin in the GnRH neuron to activate GnRH secretion. This shows the importance of intracellular and extracellular calcium. Diagram taken from Colledge, W. H., *Kisspeptin and GnRH neuronal signalling* Trends Endocrinol Metab, 2009. **20**(3): p115-121.

1.8.2. Kisspeptin signalling in cancer cells

Researchers have utilised cancer cells to investigate the signalling pathways activated by kisspeptin and gpr-54. In CHO cells, kisspeptin-10 desensitizes the chemokine receptor, CXCR4 response to SDF-1 causing a decrease in calcium release and inhibiting phosphorylation of Akt/PKB via cross-talk between the two GPCRs. This in turn blocks chemotaxis mediated via CXCR4 in these cells as mentioned previously (Navenot et al., 2005). Kisspeptin also activates the chemokine, MCIP-1 to inhibit calcineurin release in thyroid cancer cells (Stathatos et al., 2005). Whereas, Sp-1 and its co-activator DRIP-130 act to regulate *KiSS-1* expression in melanomas cells via binding to a GC-rich region between amino acids 58 to 93. This combination can decrease the invasive and migratory behaviour of the melanoma cells (Mitchell et al., 2007).

It appears that inhibition of Akt phosphorylation may be an important mechanism of kisspeptin in inhibiting migration and invasion as kisspeptin also down regulates Akt phosphorylation by tyrosine kinase receptors. Kisspeptin abolishes EGF receptor- and insulin receptor-mediated phosphorylation of Akt in an ERK1/2-dependant manner. ERK1/2-dependant mechanisms also appear important for pro-apoptotic signalling as kisspeptin via this mechanism can up-regulate apoptosis genes TNFα and FasL. Kisspeptin also increases caspase expression and cleavage of PARP, all of which are involved in apoptosis (Navenot et al., 2009). As well as Akt inhibition, reduction of MMPs has also been delineated as a mechanism for this inhibition of metastasis via kisspeptin. Fibrosarcoma HT-1080 cells stably expressing KiSS-1 have decreased MMP-9 and invasiveness. This is due to an up regulation of cytosolic IκBα, an inhibitor of NFκB, stopping its nuclear translocation. This causes a decrease in MMP-9 expression as NFκB needs to bind to the promoter of MMP-9 for activation (Yan et al., 2001). This data suggests HT-1080 cells express gpr-54. Therefore, it appears kisspeptin/gpr-54 signalling inhibits a variety of pathways to decrease the invasiveness of cancer cells (Fig. 17). However, more research is needed to delineate these mechanisms further.

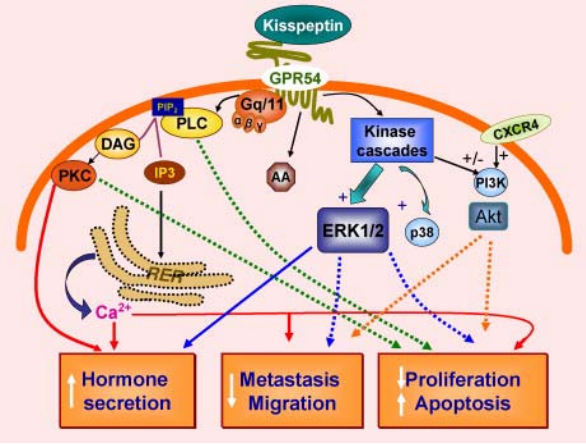

Figure 17. Signalling pathways activated by kisspeptin and gpr-54. Schematic diagram of signalling pathways proposed to be activated by kisspeptin via gpr-54. Dotted lines represent pathways that still need to be confirmed by further research. Diagram taken from Castano et al., *Intracellular signalling pathways activated by kisspeptins through gpr-54: do multiple signals underlie functional diversity?* Peptides, 2009. **30**(1): p 10-15.

GPCR mediated signalling pathways

Since a substantial part of this thesis (chapters 4 and 5) addresses the signalling pathways activated by kisspeptin at gpr-54, the following section reviews signalling pathways activated by g-protein coupled receptors, with a focus on pathways modulating cell migration.

1.9. Mitogen-activated protein kinase (MAPK) signalling pathway

MAPKs are a family of protein kinases composed of 3 major groups: 1) Extracellular-regulated kinases (ERKs), 2) jun N-terminally regulated kinases (JNKs) and 3) p38 MAPKs. Although each group has distinct functions and downstream mediators, they do share some common features. The MAPK family is activated by many receptor types such as receptor tyrosine kinases, GPCRs, cytokine and Ser/Thr kinase receptors. This then leads to the activation of a three-tier phosphorylation process to activate the MAPK. A cascade involving activation of MAPK kinase kinases (MAPKKK, MEKK or MAP3Ks) by small g-proteins such as RAS exists. MAPKKK are serine/threonine kinases which phosphorylate MAPK kinases (MAPKK or MEK), which are dual specificity kinases. MAPKKs dual phosphorylate MAPKs at a Thr-X-Tyr motif, allowing the MAPKs to phosphorylate downstream substrates on serine and threonine residues only when followed by a proline residue. These downstream targets include p90RSK1-4, MSK1/2, MNK1/2 and MAPKAP2/3 (Fig. 18) (Krishna and Narang, 2008).

1.9.1. Extracellular-regulated kinases (ERK1/2)

The first MAPK members to be characterised were ERK1 and ERK2. These are activated by the common three-tier system via the small g-protein, RAS. The main MAPKKK in the ERK1/2 pathway is raf-1, which requires binding to RAS and

phosphorylation at multiple sites for activation. This leads to activation of the MAPKK, MEK1/2 followed by ERK1/2 dual phosphorylation at a Thr-Glu-Tyr motif (Fig. 18) (Boutros et al., 2008).

Once activated ERK1/2 can target a variety of cytosolic (PLA_2, calnexin), nuclear (NFAT, Elk-1, c-fos, c-jun, p90rsk) and cytoskeletal proteins (paxillin, neurofilaments) via phosphorylation. One specific substrate of ERK1/2 is the p90rsk family of Ser/Thr kinases. Firstly, ERK1/2 docks at the C-terminal domain of p90rsk via a Leu-Arg-Gln-Arg-Arg motif allowing phosphorylation of Thr^{573} in the N-terminal kinase domain (NTKD). This activates the C-terminal kinase domain (CTKD), which is involved in autophosphorylation of Ser^{230} to create a site for PDK1. PDK1 can then phosphorylate Ser^{221} to fully activate p90rsk, to phosphorylate downstream substrates at Arg-X-Arg-X-X-pSer/Thr motifs. This co-ordinated phosphorylation is completed by the NTKD domain phosphorylating Ser^{749} within the ERK1/2 binding motif. Downstream substrates of p90rsk include transcription factors, CREB and NFAT and cytosolic proteins such as glycogen synthase kinase 3 beta (GSK3β). These then regulate processes such as cell survival, cell migration and cell cycle progression (Anjum and Blenis, 2008).

1.9.2. Jun N-terminally regulated kinases (JNKs)

JNKs were originally discovered as stress-activated protein kinases (SAPKs) but have since been shown to phosphorylate and activate members of the jun family of transcription factors. JNKS are activated via stresses such as UV radiation or DNA damage as well as via cytokines and GPCRs, mainly within the innate immune system. JNKs are also activated by three-tier phosphorylation involving a variety of MAPKKKs such as MEKK1-4, MLK2/3, Tpl-2 and TAK1 depending on the stimulus. The MAPKKK then phosphorylates MEK4 and 7, which dual phosphorylate JNK at its Thr-Pro-Tyr motif (Fig. 18). MEK7 phosphorylates Thr^{183} and MEK4 phosphorylates Tyr^{185} allowing phosphorylation of downstream substrates (Boutros et al., 2008; Krishna and Narang, 2008).

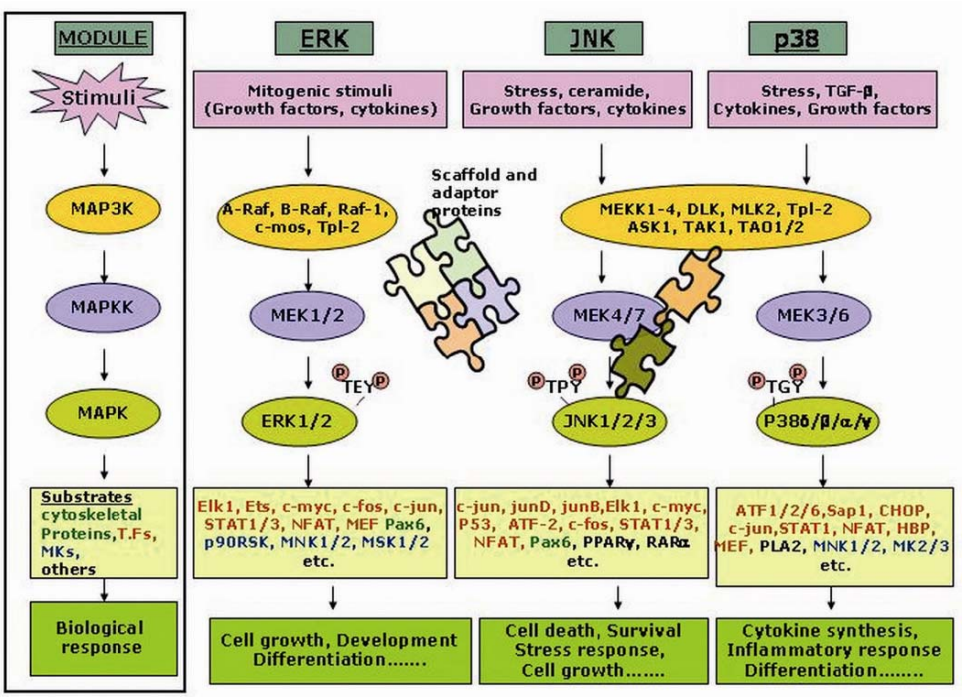

Figure 18. MAPK signalling pathway. Schematic diagram of the MAPK signalling pathway showing the three-tier phosphorylation system. Activators for each member and specific downstream mediators and biological responses are also shown. Diagram taken from Krishna and Narang, *The complexity of Mitogen-activated protein kinases (MAPKs) made simple* Cell Moll Life Sci, 2008. **65**(22): p3525-3544.

Once activated JNK targets a variety of downstream mediators, especially members of the Jun family such as c-jun, AFT-2 and c-fos. However, JNK can also modulate cytoskeletal proteins (paxillin and Tau), transcription factors (NFAT, Elk-1, c-myc) and mitochondrial proteins (Bcl-2 family). One area where JNK plays a significant role is in the control of cell migration. JNK can phosphorylate paxillin a component of focal adhesions, on Ser^{178} which has been shown via mutagenesis to increase cell migration. JNK can also phosphorylate tau preventing the assembly of microtubules (Boutros et al., 2008; Krishna and Narang, 2008).

1.9.3. p38MAPK signalling

The third major group of MAPK members is p38MAPK, which can also be activated via stress responses such as UV radiation, double stranded DNA breaks and osmotic shock as well as via cytokine receptors and GPCRs. This activation is usually via the small g-proteins Rho/Rac-1 which kick-start the three tier phosphorylation. For p38MAPKs, the MAPKKK is MLK2/3, which then activates MEK3/6 to dual phosphorylate p38MAPK at its Thr-Gly-Tyr motif (Fig. 18). This allows p38MAPK to phosphorylate downstream mediators of apoptosis, cell cycle and inflammation (Thornton and Rincon, 2009; Zarubin and Han, 2005).

p38MAPK activates a wide range of downstream targets such as cytosolic proteins (GSK3β and PPA2), transcription factors (NFAT, ATF, Elk-1 and p53) and MAPK specific substrates (MK2/3). For example, p38MAPK has been implicated as a mediator of cell survival after exposure to UV radiation and chemotherapy drugs. The mechanism involves direct phosphorylation of the kinase, GSK3β at Ser^{389}, a p38MAPK specific phosphorylation site in the C-terminus. This allows accumulation of β-catenin in the nucleus to promote cell survival (Thornton et al., 2008; Thornton and Rincon, 2009).

1.10. Nuclear Factor kappa B (NFκB) signalling

NFκB is a key mediator of inducible transcription within the innate immune response with a positive role in physiological immunity and pathological inflammation. Activation of NFκB by Toll-like receptor (TLR) adaptor proteins upon pathogen binding, promotes transcription of adhesion molecules in vascular endothelial cells leading to an initial increase of neutrophils infiltration followed by macrophage and other leukocyte infiltration (Ghosh and Hayden, 2008). NFκB is also essential in the development of innate immune cells by regulating the expression of anti-apoptotic genes

upon TLR activation. This promotes neutrophil survival to enhance the innate immune response (Hayden et al., 2006).

1.10.1. NFκBs: activation and inhibition

The NFκB family consist of five related transcription factors that can regulate inducible gene transcription. The five members are p50, p52, p65, RelA and RelB which are all encoded by separate genes. All five family members share a common amino-terminal REL homology domain (RHD) within the immunoglobulin-like domain that is known to be involved in DNA binding. Each member also contains an N-terminal IgG-like domain which confers selectivity of transcriptional regulation and a hydrophobic C-terminus for dimerization. Dimerization is essential for activation of NFκB and they can be homo- or hetro-dimers with the prototypical dimer being p50-p65. When active, dimers can then bind to κB consensus sequences (GGGRNYYYCC, where Y = unspecified pyrimidine, R = unspecified purine and N = any nucleotide) within the promoters or enhancers of target genes, causing recruitment of co-activators and co-repressors (Ghosh and Hayden, 2008; Hayden and Ghosh, 2008).

NFκB dimers are usually kept in their inactive state by inhibitors of NFκB (IκBs) in un-stimulated cells. The prototypical of these is IκBα and this mainly represses the p50/p65 dimer of NFκB. This occurs as IκBα masks the nuclear localization sequence (NLS) of the p65 subunit, but not the p50 subunit. The NLS of p50 coupled with the nuclear export sequence (NES) on IκBα causes a constant shuttling of the inactive dimer between the cytoplasm and nucleus. When cells are stimulated, IκBα is targeted for degradation by IκB kinase (IKKs) complexes. IKKβ is essential for degradation of IκBα via phosphorylation at Ser^{32} and Ser^{36} of a conserved IκB motif (DSGXXS), targeting it for proteasomal degradation. This releases NFκB dimers to localise in the nucleus and regulate gene expression (Fig.19) (Hayden and Ghosh, 2008).

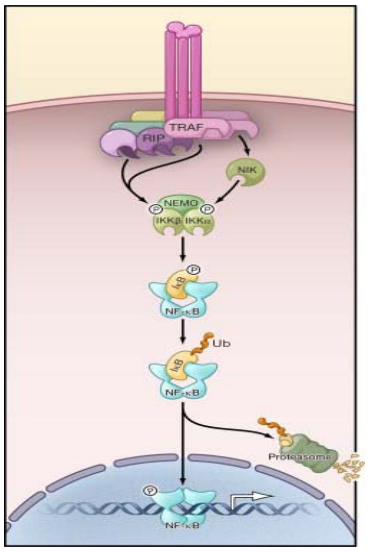

Figure 19. NF-κB signaling pathways. Following receptor ligation and recruitment of receptor proximal adaptor proteins, signalling to IKK proceeds through TRAF/RIP complexes leading to canonical NF-κB signalling. IKK activation results in IκB phosphorylation and degradation in the canonical pathway. Phosphorylated NF-κB dimers bind to κB DNA elements and induce transcription of target genes. Diagram taken from Hayden and Ghosh, *Shared principles of NF-kappaB signaling* Cell. 2008. **132**(3): p344-362.

1.11. GSK3β and β-catenin

1.11.1. β-catenin

Catenins were originally identified via co-immunoprecipitation with cadherins and have three isoforms α-catenin, β-catenin and γ-catenin (Potter et al., 1999). Catenins belong to the armadillo protein family characterised by a central domain consisting of a repeating 42 amino acid repeat (armadillo repeats) that act as a versatile protein interface. Beta-catenin contains 12 armadillo repeats which are used to form three main complexes with TCF/Lef transcription factors, APC for targeted degradation and with cadherins to form adherens junctions (Daugherty and Gottardi, 2007; Gottardi and Gumbiner, 2001). It has been proposed that β-catenin switches between cadherin and

TCF/Lef binding via Tyr^{142}, as phosphorylation of this residue by BCL-9 cause's β-catenin to dissociate from cadherins and move into the nucleus (Brembeck et al., 2004).

As well as being an integral part of the canonical WNT signalling pathway, β-catenin can also associate with cadherins at the cell membrane to form intercellular junctions which regulate cell motility. Cadherins are calcium-dependant cell adhesion glycoproteins that contain an extracellular domain, a membrane spanning domain and an intracellular domain. Beta-catenin can associate with Type 1 adhesions at adherens junctions to link E-cadherin or N-cadherin to microfilaments. The cytoplasmic cadherin domain contains a catenin-binding sequence that can interact with β-catenin forming a salt bridge between Asp^{674}/Glu^{682} of the cadherin and $Lys^{435/312}$ of β-catenin. This interaction is enhanced when E-cadherin is phosphorylated at $Ser^{684/686/692}$. Beta-catenin then binds with α-catenin which then links to the actin cytoskeleton to form a stable bond between the complex and cytoskeleton (Fig. 20). This then promotes cell adhesion and inhibits cell mobility (Gooding et al., 2004).

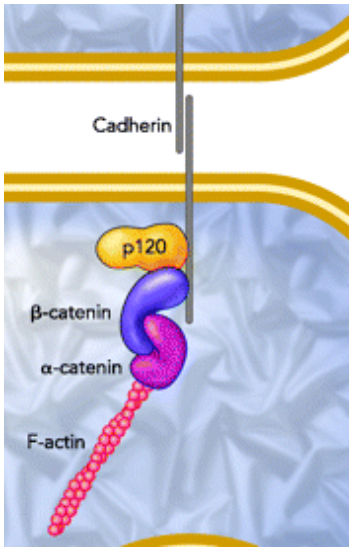

Figure 20. Adherens junctions. Diagram of an adherens junction: the connection of cadherins to the actin cytoskeleton. Beta-catenin interacts with the cytoplasmic cadherin domain and α-catenin, which then binds the actin cytoskeleton. Diagram taken from Daugherty et al., *Phospo-regulation of beta-catenin adhesion and signalling functions* Physiology (Bethesda), 2007. **22**: p303-309.

1.11.2. Glycogen Synthase Kinase 3

Glycogen synthase Kinase 3 (GSK3) is a Ser/Thr kinase that contains an activation domain in the C-terminus and an ATP-binding site (Frame and Cohen, 2001; Pearl and Barford, 2002). Of the two isoforms (α/β), GSK3β is better characterised and preferentially phosphorylates Ser/Thr residues with a pre-existing 'primed' phospho-Ser/Thr site that is four amino acids C-terminal to the GSK3β phosphorylation site. This allows for hyper-phosphorylation of substrates as most are primed at the last C-terminal Ser/Thr residue and then GSK3β can hyper-phosphorylate in a C- to N-terminal manner, with the previous residue acting as the prime for the next phosphorylation (Fig. 21). This prime site is recognised by GSK3β due to an oxyanion (phosphate)-binding site close to Val^{214} of the activation segment. This causes a conformational change via interaction with Arg^{180}/Lys^{205} of the C-terminus and Arg^{96} of the N-terminal α-helix forming a pocket that the primed Ser/Thr residue can interact with to regulate substrate specificity. This is the mechanism used to phosphorylate β-catenin within the (axin, adenomatous polyposis coli (APC), GSK3β) destruction complex of canonical WNT signalling. This targets β-catenin for ubiquitination and degradation, however, if GSK3β is inhibited β-catenin is released into the cytoplasm (Frame and Cohen, 2001; Pearl and Barford, 2002).

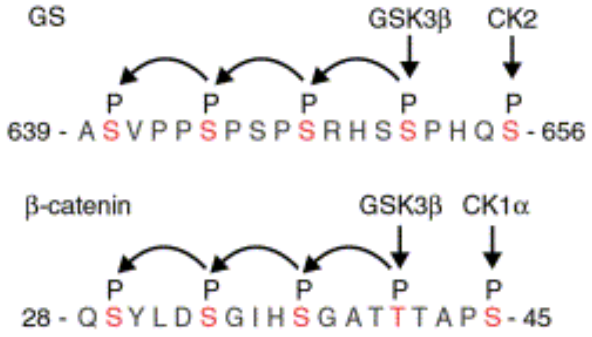

Figure 21. GSK3β hyperphosphorylation of glycogen synthase and β-catenin. Glycogen synthase (GS) and β-catenin undergo hyperphosphorylation when primed by casein kinases at the most C-terminal Ser/Thr. GSK3β phosphorylates the Ser/Thr that is four residues N-terminally to the primed site, this phosphorylation then acts as the prime for the next and so on. Diagram taken from Pearl and Bardford, *Regulation of protein kinases in insulin, growth factor and Wnt signalling* Curr Opin Struct Biol, 2002. **12**(6): p761-767.

However, GSK3β has a double life in mammals, in that it also regulates glycogen synthesis by insulin (Fig. 22), with no cross-talk between pathways. In the insulin pathway, GSK3β is also constitutively active in resting cells via Tyr^{216} phosphorylation, where it hyper-phosphorylates glycogen synthase, when primed by CK2 to inhibit glycogen synthesis. When stimulated by insulin, GSK3β is inhibited via phosphorylation at Ser^{9} by PKB/Akt through a PI(3)K-dependant pathway. This promotes dephosphorylation of glycogen synthase and up-regulates glycogen synthesis (Pearl and Barford, 2002). This is critical for normal glycogen synthesis by insulin in adipose tissue (McManus et al., 2005).

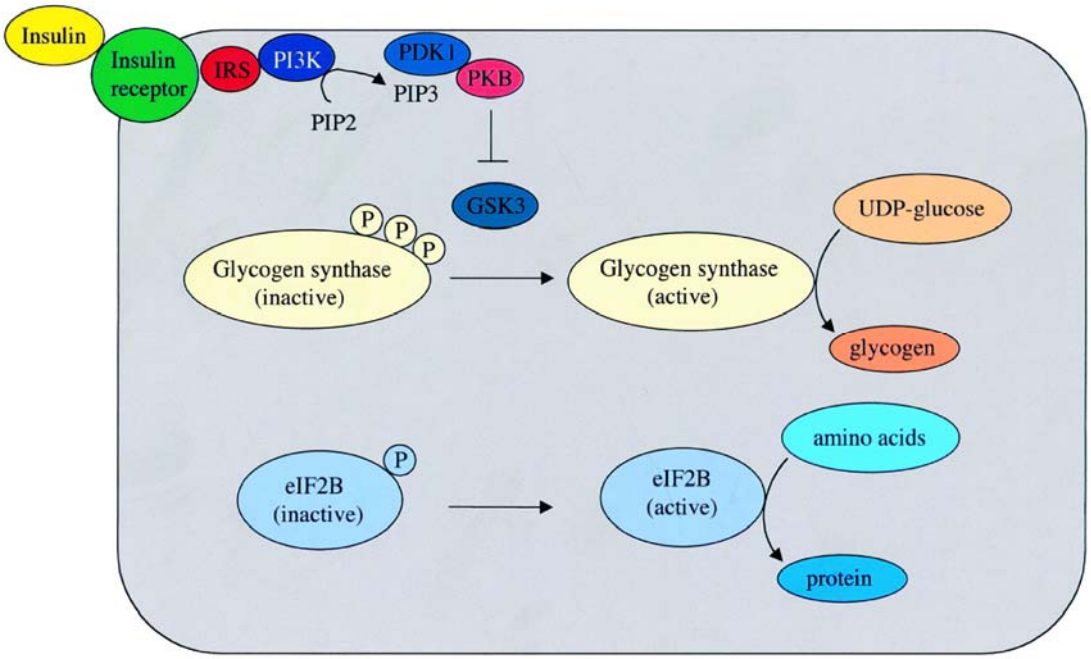

Figure 22. Insulin signalling. Diagram of signalling via the insulin receptor to inhibit GSK3β via Ser^{9} phosphorylation by PKB/Akt via PI(3)K pathway. This allows release of glycogen synthase to produce glycogen. Diagram taken from Frame and Cohen, *GSK3 takes centre stage more than 20 years after its discovery* Biochem J, 2001. **359**(Pt 1): p1-16.

PKB/Akt is not the only kinase that can phosphorylate this Ser^{9} residue, the ERK1/2 specific substrate p90rsk can also phospho-inhibit GSK3β via this residue but is known

to target β-catenin to the plasma membrane to interact with cadherins, so increases adhesion and inhibits migration. Inhibition by p90rsk is primed on GSK3β via ERK1/2, which can dock at an FKFP motif and phosphorylate the priming residue Thr^{43} (Fig. 23). This inhibition is usually regulated by growth factors such as fibroblast growth factor (FGF) or epidermal growth factor (EGF) (Ding et al., 2005; Frame and Cohen, 2001; Torres et al., 1999). It is also known that PKA, PKC and p70s6k kinases can also phosphorylate this residue (Joshi et al., 2007). Inhibition of GSK3β by Ser^9 phosphorylation works in a similar manner to GSK3β hyperphosphorylation as this phosphorylation creates a primed pseudo-substrate in the N-terminus. This can occupy the oxyanion-binding pocket and compete with primed substrates, however GSK3β is not then hyperphosphorylated as the residue four positions N-terminally is a proline, so this keeps GSK3β inhibited (Frame and Cohen, 2001; Pearl and Barford, 2002).

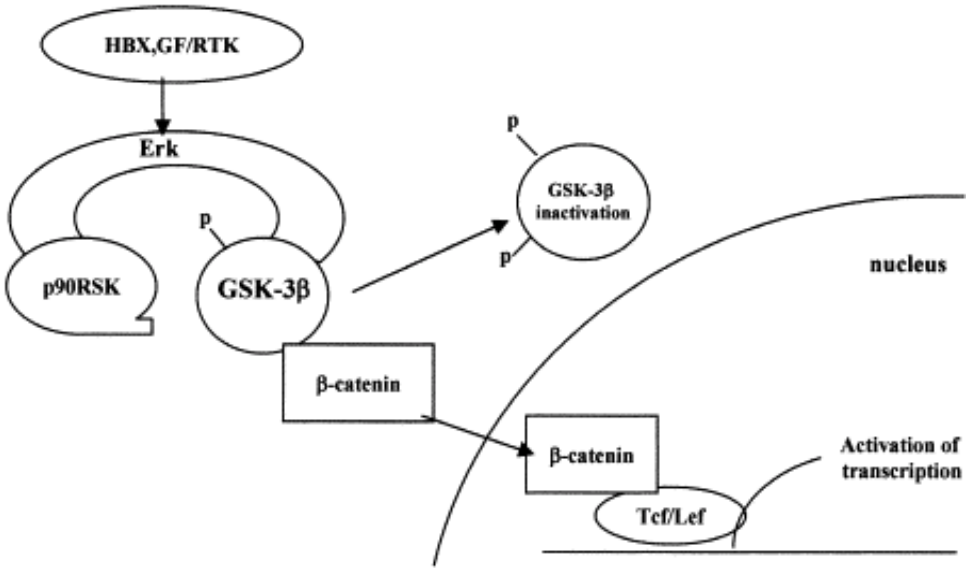

Figure 23. Regulation of GSK3β by p90rsk. Model of GSK3β inhibition by p90rsk phosphorylation of Ser^9 when primed by ERK1/2 allowing release of β-catenin. Diagram taken from Ding et al., *Erk associates with and primes GSK-3beta for its inactivation resulting in up regulation of beta-catenin* Mol Cell, 2005. **19**(2): p159-170.

1.11.3. Regulation of GSK3β/β-catenin by g-protein coupled receptors

More recently, it has been discovered that GPCRs can also regulate GSK3β and β-catenin. In 2001, the first GPCR shown to regulate these molecules was the prostanoid receptor FP_B activated by the prostaglandin, $PGF_{2\alpha}$. GPCR activation can cause dephosphorylation and nuclear accumulation of β-catenin, resulting in activation of TCF/Lef transcriptional activity (Fujino and Regan, 2001). In reproductive tissues, GnRH receptor activation can inhibit GSK3β, stabilize β-catenin in the nucleus and activate TCF/Lef transcription of the pro-apoptotic genes c-jun, c-myc and Fra-1 (Gardner et al., 2007).

1.12. Signalling at focal adhesions

Integrins are transmembrane receptors that can bind to proteins within the extracellular matrix (ECM) such as collagen and fibronectin. The integrins convey signals about the adhesion status of the cells to regulate cell survival, proliferation and migration. Integrins form heterodimers consisting of α and β subunits localized within the plasma membrane where their short cytoplasmic domains functionally link changes in the ECM to the actin cytoskeleton inside the cell. The sites where integrins connect to the cytoskeleton are called focal adhesions and these are comprised of a multiprotein complex of scaffolding and signalling proteins (Fig. 24). This complex contains protein tyrosine kinases and these are usually activated via integrin clustering, although this can also occur via GPCR, cytokine and growth factor receptor signalling (Lim et al., 2008).

An important tyrosine kinase in focal adhesions is focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase that is targeted to focal adhesions upon integrin clustering. FAK consists of an N-terminal FERM domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain (Fig. 25). The FAT domain is where FAK indirectly links to integrins via the binding protein, paxillin. Paxillin binds to the

cytoplasmic domain of integrins and to vinculin, a focal adhesion associated protein. Paxillin can also interact with p21-activated protein kinase (PAK) when phosphorylated at $Tyr^{31/118}$; this can then phosphorylate MEK to up regulate MAPK signalling. FAK also contains proline-rich domains that bind Src-homology 3 (SH3) containing proteins such as p130Cas and conserved phosphorylation sites which can serve as SH2 domains to bind Src, PI(3)K or adapter proteins such as Grb2 (Golubovskaya et al., 2009; Lim et al., 2008; Parsons, 2003).

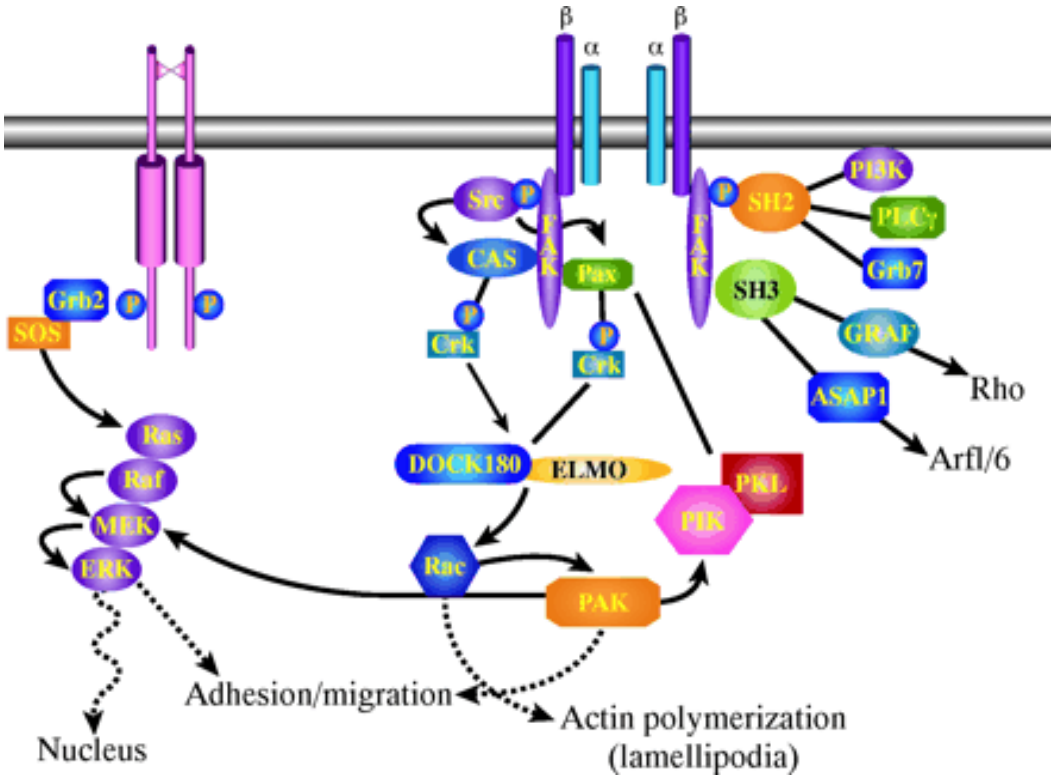

Figure 24. Signalling at focal adhesions. Diagram showing the multiprotein complex involved in focal adhesion signalling involving integrins, FAK, Paxillin, Src and p130Cas. This complex can facilitate downstream signalling to MAPKs, PAK and PI(3)K to effect actin polymerization and cell migration. Diagram taken from Parsons, J.T., *Focal adhesion kinase: the first ten years* J Cell Sci, 2003. **116** (Pt. 8): p1409-1416.

FAK is activated in response to integrin clustering via autophosphorylation of Tyr^{397}, creating a SH2 domain for binding with Src. Full activation of FAK can then occur via phosphorylation of $Tyr^{567/577}$ by Src and FAK can enhance Src activity via phosphorylation at Tyr^{418}. FAK-Src complexes can bind to paxillin and p130Cas at focal adhesions or regulate downstream signalling cascades. For example, if Src phosphorylates Tyr^{925} of FAK this promotes binding of Grb2 and activates the MAPK cascade. Inhibition of FAK occurs via its FERM domain, which can bind to the kinase domain and inhibit via an auto-inhibitory mechanism. This causes steric inhibition of target proteins at the catalytic cleft and Tyr^{397}. This inhibition is released when proteins and phospholipids bind to the FERM domain (Cohen and Guan, 2005; Lim et al., 2008; Parsons, 2003).

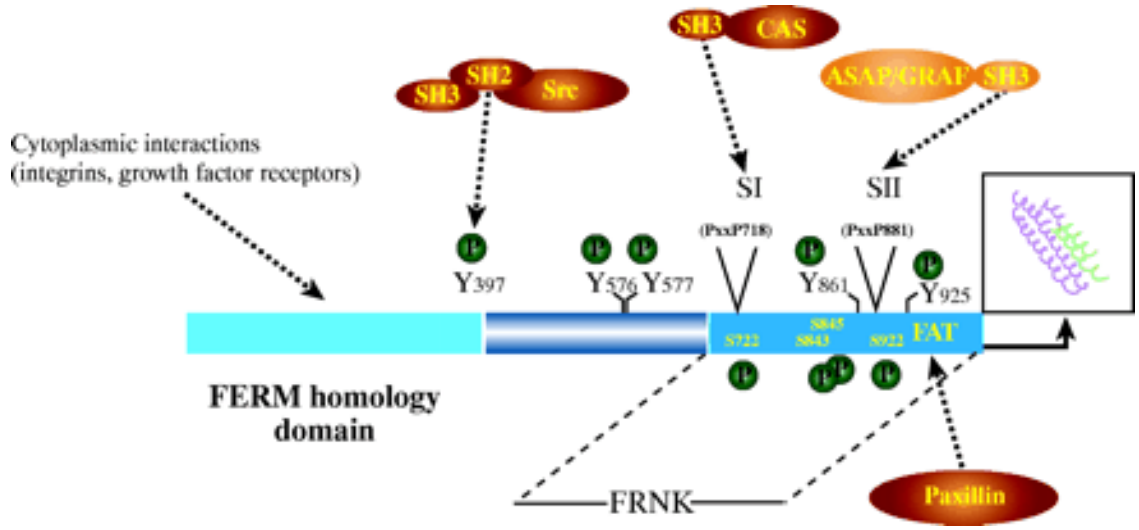

Figure 25. Focal Adhesion Kinase. Schematic Diagram of focal adhesion kinase representing the cytoplasmic FERM domain, the kinase domain containing three phosphorylation sites and the N-terminal domain (also known as FRNK domain) containing the FAT domain and two phosphorylation sites. Src binds to the auto-phosphorylated Tyr^{397} to then phosphorylate the other two sites within the kinase domain. Paxillin can then bind to the FAT domain and p130Cas to the N-terminal domain. Diagram taken from Parsons, J.T., *Focal adhesion kinase: the first ten years* J Cell Sci, 2003. **116** (Pt. 8): p1409-1416.

1.13. Summary

In this chapter, kisspeptin/gpr-54 and MAPK, NFκB, β-catenin, GSK3β and FAK signalling pathways have been reviewed. A review of the recent literature suggests that kisspeptin and gpr-54 are critical for hormonal control of reproductive tissue function via regulation at multiple levels including initiation of puberty, steroid feedback to the hypothalamus and within different cell types of the ovary and placenta. However, the evidence implicating the importance of kisspeptin and gpr-54 in these tissues is so far indirect, creating a need for antagonists of this system to determine direct regulation of these pathways via gpr-54. This will be addressed as part of the research within this thesis where I aim to define the structural elements of kisspeptin required to bind to and activate gpr-54 using synthetic peptide analogues. These analogues will also be used to screen for *in vitro* receptor antagonism and then selected analogues will be evaluated via *in vivo* models.

This review covered the signalling pathways activated by kisspeptin known to date, including signalling pathways involved in the regulation of cell migration. The literature on signalling by kisspeptin is limited and needs further investigation and will therefore be encompassed within the remainder of the research in this thesis. Here I aim to delineate the signalling pathways activated by gpr-54 *in vitro* using stably transfected CHO and GT_{1-7} cells and a trophoblast cell line. The effects of kisspeptin and analogues on cell migration and the pathways involved will also be examined.

Chapter Two

Materials and Methods

2.0. Introduction

This chapter details the materials and laboratory techniques used throughout this research. The source of all materials is provided and any work not carried out by myself acknowledged. All chemicals where a manufacturer is not stated in the text were purchased from Sigma.

2.1. Materials

Human kisspeptin-10 and peptide analogues 186-191, 200-203, 206-213, 228-248 and 271-278 were custom synthesized by EZBiolabs, USA. Chinese hamster ovary (CHO) cells stably expressing gpr-54 were obtained from Prof. G Vassart (Univ. Brussels); mouse GT_{1-7} and $L\beta T_2$ cells were obtained from Dr Pamela Mellon (Univ. California, USA); human HTR8SVneo cells were obtained from Dr G. Pare (Queen's Univ., Ontario, Canada); HUVEC cells were obtained from Dr Rebecca Reynolds (Edinburgh, UK); BeWo choriocarcinoma cells were obtained from the American Type Culture Collection (ATCC) and COS-7, JAR, JEG-3, MCF-7 and rat GH3 cells were obtained from the European Collection of Cell Cultures (ECACC). The Src inhibitor PP2, the PI(3)K inhibitor LY294002, the PKC inhibitor Go6983, a second PKC inhibitor Ro-31-8220, the specific MEK inhibitor PD98059, the PI(3)K specific inhibitor PI-103, the specific Src-Inhibitor 1 and the specific EGFR inhibitor AG1478 were obtained from Calbiochem (Nottingham, UK). A specific Akt1/2 inhibitor was obtained from Sigma, UK. The specific GSK3β inhibitor CT99021 and the specific p90rsk inhibitor BI-D1870 were obtained from Dundee University, UK. The specific $G_{q/11}$ inhibitor YM254890 was kindly provided by Dr Masatoshi Taniguchi (Astellas Pharmaceuticals Inc, Japan). The RGDS and RGES tetrapeptides were obtained from Sigma, UK. D-[*myo*-3H]-inositol was obtained from GE Healthcare (Buckinghamshire, UK). The mouse gpr-54 cDNA expression construct was produced in the laboratory by Dr Kevin Morgan. The pSRE-luciferase and renilla constructs were obtained from Dr Elena Faccenda (Edinburgh).

2.2. Preparation of plasmid DNA

A scrape of bacteria from a glycerol stock was added to a starter culture of 10ml fresh terrific broth medium containing selective antibiotic (Ampicillin – stock solution 50mg/ml, Sigma) and grown for a period of 10 hours. 2ml were then used to inoculate a 250ml terrific broth medium culture and this was grown for a further 16 hours. Plasmid DNA was purified using Qiagen maxi-preparation columns and eluted using TE Buffer (10mM Tris-HCL (pH8), 1mM EDTA) according to the manufacturer's instructions. DNA concentration and quality was determined using the nanodrop (Nanodrop technologies, Delaware).

2.3. Preparation of glycerol stocks of transformed bacteria

Glycerol stocks of transformed E.coli were made by adding 400ul of 80% glycerol to 600ul of bacterial culture that had been grown for a period of 10 hours. Vials were inverted to mix the glycerol and stored at -80ºC. To recover the bacteria, a sterile inoculating loop was used to scrape the surface of the frozen culture, keeping the bacteria on dry ice. The plasmid DNA was then prepared as described in section 2.2.

2.4. Agarose gel electrophoresis

1% agarose gels were prepared in TAE buffer (40mM Tris, 320mM acetic acid, 1mM EDTA, pH 7.2). Plasmid DNA and PCR products were separated at 120V for 30 minutes, stained with ethidium bromide and visualised under ultraviolet light using a transilluminator. To obtain size of fragment, either a 1kb DNA or DNA step ladder was utilized (Promega, Madison, WI, USA)

2.5. Design and synthesis of peptide analogues

Peptide analogues were designed based on the sequence of kisspeptin-10, the final ten C-terminal amino acids of kisspeptin. Firstly, kisspeptin-10 was systematically truncated at the N-terminus to 5 and 7 amino acid lengths in order to determine the shortest C-terminal peptide capable of binding and activating the receptor. Then amino acid substitutions of the residues within the truncated and full length peptides were performed, firstly the C-terminal amino acids were substituted, then the N-terminal residues to find amino acids important for receptor binding or activation. The peptide analogues were synthesized by EZBiolabs, USA. Peptide analogues for preliminary studies were synthesized to a purity of >80% (range 83-99% measured by HPLC) and peptides 234, 271 and 273, which were selected for detailed studies were synthesized at a purity of >95%. The authenticity of peptides was confirmed by mass spectrometry. Once synthesized analogues were tested for antagonism and agonism using the methods described.

2.6. Cell Culture

2.6.1. Cryopreservation and thawing of cell lines

Stocks of each cell line were stored at -196ºC under liquid nitrogen in cryoprotectant (10% dimethylsulphoxide (DMSO) (v/v) in fetal calf serum). Cells were recovered from liquid nitrogen store and rapidly warmed to 37ºC. They were then gently re-suspended in media and seeded into flasks. Frozen stocks were preserved by banking cells for the first few passages after they were resuscitated. Confluent cultures were passaged as described below (section 2.6.2), and cells collected by centrifugation at 2000rpm for 3 minutes. The medium was then decanted and the cell pellet gently re-suspended in cryoprotectant, aliquoted into vials and frozen at -80ºC in a cryo 1ºC freezing container (Nalgene). Vials were then transferred to liquid nitrogen for long term storage.

2.6.2. Stable and immortalized cell lines

Chinese hamster ovary cells stably expressing the human gpr-54 receptor (CHO/gpr-54); $L\beta T_2$ cells stably expressing mouse gpr-54 ($L\beta T_2$/gpr-54) and GT_{1-7} cells stably expressing the mouse gpr-54 receptor (GT_{1-7}/gpr-54) were maintained in Dulbecco's modified Eagles medium (DMEM, Sigma) supplemented with 10% fetal calf serum, 2% Glutamine, 1% Penicillin (10,000units/ml)/Streptomycin (10,000mg/ml) and 500µg/ml geneticin (G418; PAA Laboratories, Somerset, UK) at 37ºC in a humidified 5% CO_2 atmosphere. COS-7, GH3, Ishikawa, JEG-3, JAR and MCF-7 cells were maintained as above minus the geneticin. BeWo choricarcinoma cells were maintained in F-12 HAM medium (Invitrogen) supplemented with 10% fetal calf serum, 2% Glutamine, 1% Penicillin (10,000units/ml)/Streptomycin (10,000mg/ml) at 37ºC in a humidified 5% CO_2 atmosphere. HTR8SVneo immortalized trophoblast cells were maintained in RPMI 1640 medium (Invitrogen) containing glutamine and supplemented with 5% fetal calf serum and 1% Penicillin (10,000units/ml)/Streptomycin (10,000units/ml) at 37ºC in a humidified 5% CO_2 atmosphere. HUVEC cells were maintained in EGM2 medium (Lonza) supplemented with hydrocortisone, VEGF, rhEGF, rhFGF, R3-IGF-1, heparin, ascorbic acid, GA-1000 and 2% fetal bovine serum at 37ºC in a humidified 5% CO_2 atmosphere.

Cells lines were routinely passaged twice weekly by enzymatic dispersal with trypsin. The medium was removed from confluent cells and cells washed with Dulbecco's phosphate buffered saline (PBS; Lonza). Then 2ml of 1x trypsin were added to each 162cm^2 flask (Corning) and flasks returned to incubator at 37ºC for 3-5 minutes until cells were easily detached. 8ml of media were then added to inhibit the trypsin and all cell lines were routinely split 1:5 with appropriate antibiotics added. Flasks were then returned to the incubator at 37ºC in a humidified 5% CO_2 atmosphere. Dispersed cells were then diluted 1:10 in PBS and counted using a Nebauer haemocytometer. Cells were counted in all four counting areas and an average taken to give the cell number x 10^5 per ml. The cells were then seeded onto plates/dishes for experiments as needed.

2.7. Transfection techniques

2.7.1. Electroporation

COS-7 cells were seeded into 150cm^2 dishes (Corning), to be 80% confluent at time of transfection. Confluent cells had medium removed and cells were washed three times with PBS. Then 2ml of 1x trypsin were added to each dish and dishes returned to incubator at 37ºC for 10 minutes until cells were easily detached. 8ml of medium were then added to each dish to inhibit the trypsin. Dispersed cells were collated and then collected by centrifugation at 2000 rpm for 3 minutes. The medium was decanted and the pellet gently re-suspended in 25ml of Optimem-1 (Gibco, Invitrogen, UK) electroporation medium. The cells were again collected by centrifugation at 2000 rpm for 3 minutes. The medium was decanted and the pellet re-suspended in 0.7ml/dish Optimem-1 electroporation medium (Gibco). Then 0.7ml cell suspension was added to each pre-chilled electroporation cuvette (Bio-rad) containing 10ug DNA and mixed with gentle pipetting. Each cuvette was placed in the electroporation shocking chamber (Bio-rad Gene Pulsar Xcell) and pulsed at 0.22kV and 960µF with an average time constant of 21ms. Cuvettes were then left to settle for 10 minutes at room temperature. Transfected cells were then seeded into 12-well plates at 1x10^5 cells/well for experiments as needed.

2.7.2. Chemical Transfection – Fugene 6 reagent

Cells were seeded at 1x10^5 cells/well in 12-well plates (Costar) as needed. The transfection medium was prepared as follows for each 12-well plate and left to equilibrate at room temperature for 30 minutes:

10µg DNA
316.6ul Fugene 6 transfection reagent (Roche)
9.5ml Optimem-1

After 30 minutes, 9.5ml of complete media was added to transfection medium to stop the reaction. Cells then had medium removed and were washed once with PBS. Then 1ml/well of Fugene 6 transfection medium was added to cells and plates incubated overnight at 37ºC in a humidified 5% CO_2 atmosphere.

2.7.3. Chemical Transfection – Superfect reagent

Cells were seeded at $1x10^5$ cells/well in 12-well plates as needed. The transfection medium was prepared as follows for each 12-well plate and left to equilibrate at room temperature for 30 minutes:

10µg DNA
240ul Superfect transfection reagent (Qiagen)
2.3ml DMEM

After 30 minutes, 2.3ml complete media was added to the transfection medium to stop the reaction. Cells then had medium removed and were washed once with PBS. Then 400ul/well of Superfect transfection medium was added to cells and cells incubated at 37ºC in a humidified 5% CO_2 atmosphere for 4 hours. Transfection medium was then removed and cells were washed once with PBS. Lastly, 2ml/well of culture medium was added to cells and cells incubated overnight at 37ºC in a humidified 5% CO_2 atmosphere.

2.8. Preparation of pharmacological inhibitors and ligands

Stock solutions of inhibitors were prepared in DMSO or as per manufacturers' instructions, aliquoted and stored at 4ºC or -20ºC. Stock solutions of kisspeptin-10 and kisspeptin analogues were prepared in 20% polyethylene glycol, aliquoted and stored at -20 ºC. After aliquots were defrosted they were diluted further in 20% polyethylene glycol as needed.

2.9. Whole cell receptor binding

Kisspeptin-10 was prepared at 1:100 dilution in HEPES modified DMEM supplemented with 0.1% Bovine Serum albumin and ^{125}I-labelled Kisspeptin-10 (100,000 cpm/0.5mls). Cell monolayers seeded at $1x10^5$ cells/well in 12-well plates were placed on ice and exposed to 0.5ml peptide/well (10pM-10uM); the cells were then incubated at 4ºC for 4 hours. After 4 hours, cells were washed twice with ice cold PBS (with Calcium and Magnesium) and then 0.5ml 0.1M Sodium Hydroxide (NaOH) were added to cells for 20 minutes, while shaking. Lysates were transferred to plastic tubes and bound radioactivity counted on the Wallac 1470 Wizard gamma counter (PerkinElmer and Analytical Sciences (UK) Ltd, Buckinghamshire, UK) for 60 seconds.

2.10. Inositol phosphate (IP) assay

Cell monolayers seeded at $1x10^5$ cells/well in 12-well plates as needed, were labelled overnight with 1μCi/ml D-[*myo*-3H]-inositol in inositol-free DMEM supplemented with 1% dialyzed fetal calf serum, 2% glutamine and 1% penicillin/streptomycin. Cells were then incubated in 0.5ml HEPES modified DMEM supplemented with 1% Penicillin/Streptomycin and 10mM Lithium Chloride (LiCl) for 30 minutes at 37ºC. Cells were then stimulated with 0.5ml/well kisspeptin-10 (10pM-10uM) ± peptide analogues for 1 hour at 37ºC, then placed in 1ml of 10mM formic acid at 4ºC for 1 hour. Lysates were then transferred to plastic tubes containing 0.5ml Dowex AG1-X8 ion exchange resin (Bio-rad). The resin was then washed with 1ml of water followed by 1ml of 60mM ammonium (NH_4) formate/5mM sodium tetraborate and finally 1ml of 1M ammonium (NH_4) formate/0.1M formic acid. Then 800μl of the radioactive solution were transferred to scintillation vials (Zinsser analytic) containing 2.5ml scintillation fluid (Fischer Scientific) and radioactivity counted on a Wallac Microbeta Trilux β-counter (PerkinElmer and Analytical Sciences (UK) Ltd, Buckinghamshire, UK) for 60 seconds.

2.11. Fluorescence Intensity Plate Reader (FLIPR) calcium 4 assay

Cell monolayers were plated in black, clear bottomed 96-well view plates (PerkinElmer, UK) overnight. Cells were then incubated in serum-free media at 37ºC for 1 hour prior to stimulation with FLIPR calcium 4 reagent (Molecular Devices, USA). Cells were then incubated for 1 hour at 37ºC while ligands were prepared in a separate flat bottomed 96-well plate (Costar). Cells were then tested for intensity of fluorescence on the Novostar (BMG Labtech, UK) over 60 seconds with ligands being added 1:10 after 11 seconds in reading mode.

2.12. Preparation of whole cell lysates and nuclear/cytoplasmic fractions

2.12.1. Whole cell lysates

Cells were seeded at a density of $3x10^5$ cells/well in 6-well plates (Costar) and allowed to attach overnight before being incubated in serum-free media (DMEM, 2% Glutamine, 1% Penicillin/Streptomycin and 10mM HEPES) for 16 hours. Agonist stimulations were performed in serum-free media at 37ºC after appropriate incubation with chemical inhibitors, as described in figure legends. After stimulation, cell monolayers were placed on ice, washed with ice-cold PBS and lysed in a Nonidet-P40-based solubilisation buffer (250mM NaCl, 50mM HEPES, 0.5% Nonidet P-40, 10% glycerol, 2mM EDTA pH 8.0) supplemented with 1mM Sodium orthovanadate, 1mM phenlymethlsulphonyl fluoride and 1mg/ml leupeptin. Cell lysates were clarified by centrifugation at 13000 rpm for 10 minutes.

100ul clarified whole cell lysates were mixed with an equal amount of 2x Laemmli sample buffer (LSB) and samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To give a measurement of the size of proteins pre-stained broad range SDS-PAGE standards were utilized (Bio-Rad). The gels were 4-20% Tris-

glycine gels (invitrogen) and a Novablot semi-dry transfer apparatus (Amersham Pharmacia Biotech) was utilised. Polyvinylidene difluoride (PVDF) membrane (NEN Life Sciences, Buckinghamshire, UK) was soaked in 100% ethanol for 5 minutes, followed by 3x 5 minute washes in distilled water and then equilibrated in semi-dry transfer buffer (20mM Tris, 192mM glycine, 20% methanol and 0.1% SDS). Blotting paper pads were soaked in semi-dry transfer buffer and 1 pad place on either side of the gel on the transfer apparatus: blotting paper, PVDF membrane, gel then blotting paper. Air bubbles were removed by applying pressure with a glass tube and then the cathode plate was placed on top. The proteins were then transferred to the membranes at 0.2A for 1 hour. PVDF membranes were then blocked in 4% BSA (50mM Tris-HCl, pH 7.0, 0.05% Tween-20 and 0.05% Nonidet-P40) blocking solution for 1 hour, while shaking on a rocker. The blocking buffer was then discarded and primary antibody added for either 1 hour or left overnight.

2.12.2. Nuclear/cytoplasmic fraction lysates

Cells were seeded at a density of $3x10^5$ cells/well in 6-well plates and allowed to attach overnight before being incubated in serum-free media for 16 hours. Agonist stimulations were performed in serum-free media at 37ºC after appropriate incubation with chemical inhibitors, as described in figure legends. After stimulation, cell monolayers were placed on ice, washed with ice-cold PBS and lysed in a Nonidet-P40-based solubilisation buffer (250mM NaCl, 50mM HEPES, 0.5% Nonidet P-40, 10% glycerol, 2mM EDTA pH 8.0) supplemented with 1mM Sodium orthovanadate, 1mM phenlymethlsulphonyl fluoride and 1mg/ml leupeptin. Cell nuclei were crudely extracted from solubilised lysates by centrifugation at a low spin of 2900 rpm for 10 minutes. The cytoplasmic fraction was then removed to a fresh eppendorf and further clarified by centrifugation at 13000 rpm for 10 minutes (approximate concentration 1mg/ml of total protein). Then 100ul of clarified cytoplasmic lysate was mixed with an equal amount of 2x LSB in fresh eppendorf tubes.

The nuclear pellet was washed with Nonidet-P40-based solubilisation buffer four times, to remove contaminating cytoplasmic proteins. The nuclear pellet was then re-suspended in 100ul of Nonidet-P40-based solubilisation buffer and the nuclear membrane ruptured by sonification. Insoluble material was sedimented by centrifugation at 13000 rpm for 10 minutes. Then 60ul of clarified nuclear lysate was mixed with an equal amount of 2x LSB in fresh eppendorf tubes. Both nuclear and cytoplasmic lysates resolved by SDS-PAGE and then processed the same as for whole cell lysates (section 2.12.1.).

2.13. Immunoblotting and detection

Immunoblotting was performed at a 1:1000 dilution. Immunoblotting of pERK1/2, pJNK, p38MAPK (New England Biolabs, USA), pNF_KB, pAkt (T308), β-catenin, pGSK3β (Ser9), pFAK (Y925; Cell Signaling Technology, USA), pSrc (Y418; Invitrogen Ltd, Paisley, UK), Rac-1, pIκBα, RhoA (Santa Cruz Biotechnology Inc., USA) and RAS (Upstate Biotechnology, USA) were visualised by rabbit anti-human antibodies. Cdc42 (Santa Cruz Biotechnology Inc., USA) was visualized by mouse anti-human antibodies. Each antibody was used for a maximum of 10 times then replaced. Immunoblots were then washed three times for 10 minutes with 1x TBS-T to remove excess antibody. The proteins were then visualized by addition of a 1:10000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit or anti-mouse IgG as a secondary antibody for 1 hour. The antibodies raised against ERK1/2 were used as a loading control. Immunoblots were then again washed with 1x TBS-T to remove excess secondary antibody. Each alkaline phosphatase labelled protein was visualized using an enzyme-linked chemifluorescence reaction (GE Healthcare, Buckinghamshire, UK) and quantified using a Typhoon 9400 Phosphoimager and Imagequant TL software (Molecular Dynamics, Amersham Biosciences).

After immunoblots were scanned, membranes were washed in 40% methanol for 30 minutes to remove the ECF substrate and then washed three times with 1x TBS-T for 10 minutes. Antibodies were then stripped from the membrane by incubating in 10ml of 25mM Tris-HCl (pH 7), 8% (w/v) SDS, 0.72M β-mercaptoethanol for 30 minutes at 80ºC followed by three 10 minute washes with 1x TBS-T. The membranes were then either probed with another antibody or dried and stored at room temperature.

2.14. Dual light reporter gene luciferase assay

Transient transfections were performed using Superfect reagent for CHO/gpr-54 and $L\beta T_2$/gpr-54 cell lines. Fugene 6 reagent and electroporation were also utilized for the CHO/gpr-54 cell line. Cells were seeded at $1x10^5$ cells/well in 12-well plates, as needed and the next day transfected with a SRE-luciferase reporter construct as well as a Renilla luciferase construct to control for transfection efficiency. Cells were then placed in starving media, overnight before stimulations were performed for 24 hours. Luciferase activity was assayed using a Dual-light luciferase assay kit (Promega) in a FLUOstar Optima luminometer (BMG Lab technologies). Luciferase activity was expressed in arbitrary units relative to activity observed in non-stimulated control. The results were then expressed as fold over basal as indicated in figure legends.

2.15. RNA Purification

Confluent cells monolayers had media removed and were washed once with PBS. The cells were then lysed with Tri-reagent (Phenol; Guanidine thiocynate), which also inhibits any ribonucleases. Once detached cells were placed in fresh eppendorf tubes and 200ul chloroform added, causing the mixture to separate into different phases with RNA at the top, then a protein layer and DNA at the bottom. The RNA layer was then isolated and placed in a fresh eppendorf tube to which 600ul isopropanol was added along with glycogen. The RNA was then precipitated at 10000 rpm for 10 minutes. The pellet was then frozen at -80ºC until needed.

2.16. Reverse Transcription for real time - polymerase chain reaction (RT-PCR)

RNA precipitate was thawed at room temperature and then clarified at 13000 rpm for 10 minutes. The supernatant was decanted and the pellet gently re-suspended in 1ml of 70% ethanol (700ul ethanol and 300ul Diethylpyrocarbonate (DEPC) water). This was also clarified at 13000 rpm for 10 minutes and the supernatant decanted. The pellet was then dried at room temperature. The RNA pellet was then dissolved in DEPC water to give 1µg/ml. The following reagents were then mixed:

3.5µl Water
2.5µl Random Primers
1.0µl 10mM dNTPs
5.0µl RNA

The master mixture was heated to 65ºC for 5 minutes in the PCR machine (PCRSprint, Thermohybaid, UK), then cooled to room temperature and clarified at 13000 rpm for 1 minute. Then the following reagents were added to the above mastermix:

4.0µl 5x Buffer
2.0µl 0.1M DTT
1.0µl RNAse inhibitor
1.0µl Reverse transcription enzyme

The master mixture was then mixed and incubated at 42ºC for 1 hour in the PCR machine for reverse transcription to occur. The reaction was then stopped by heating the master mixture to 95ºC for 3 minutes before being placed on ice. Finally the reverse transcription cDNA product was mixed then diluted with 80µl DEPC-water. The cDNA was then kept at -80ºC until needed for RT-PCR.

2.17. RT-PCR

The PCR master mixture was prepared as follows:

33.25µl water
5.0µl 10x Buffer
2.5µl DMSO
2.0µl Magnesium Chloride
1.5µl Forward Primer
1.5µl Reverse Primer
1.75µl 10mM dNTPs
2.0µl cDNA
0.5µl enzymes

The master mixture was then mixed and heated to 94ºC for 90 seconds then left to progress through the following PCR cycle for 30-40 repeats:

94ºC for 30 seconds to denature the cDNA
55ºC for 60 seconds to allow attachment of primers
72ºC for 60 seconds for translation to occur

Once complete the PCR product was heated to 72ºC for 10 minutes to complete the reaction. The PCR products were then cooled at room temperature then 20ul of PCR product was added to 2ul blue dye. This was then loaded onto an agarose gel and run at 120V for 30 minutes and visualised under ultraviolet light using a transilluminator. To obtain size of fragment, either a 1kb DNA or DNA step ladder was utilized (Promega, Madison, WI, USA). The primers used for RT-PCR were a mouse gpr-54 forward primer - ATG GCC ACC GAG GCG ACA TTG GCT and reverse primer - AAG TGA GGC AGT GCG TTC ACT CTG.

2.18. Cell Migration assay

Confluent cell monolayers were scratched with a yellow pipette tip and were then washed three times with PBS at 37ºC to remove any loose cells. Cells were then stimulated with kisspeptin with or without inhibitors. Cells were incubated at 37ºC with 5% CO_2 in air for 22hrs. Cell scratches were photographed at 0 and 22hrs and the width of the scratch recorded at each using the Axiovert 200 microscope. Migration was evaluated by the distance refilled between the furthest migrated cell and the scraped edge on both sides.

2.19. Silver Nitrate gel staining

15µl of whole cell lysate samples were mixed with an equal amount of sample buffer (5% Glycerol, 10mM Tris and bromophenyl blue). Samples were then separated by PAGE using 4-20% Tris-glycine gels at 0.45mA for 45 minutes. Gels were then soaked in fixing solution (75ml EtOH, 25ml acetic acid and 150ml water) for 2 hours, while shaking on a rocker. The gel was then moved to a sensitizing solution (75ml EtOH, 10ml sodium thiosulphate, 17g sodium acetate and 150ml water) for a further 2 hours, while shaking on a rocker. The gel was then washed five times for 15 minutes with water prior to the silver nitrate solution (25ml silver nitrate, 225ml water ant 0.1ml formaldehyde) being added for 2 hours under constant shaking on a rocker. After 2 hours, the gel was given four 1 minute washes with water then a developing solution (6.25g sodium carbonate, 250ml water and 0.2ml formaldehyde) was added, while shaking on a rocker, for 6 minutes or until bands became detectable. The reaction was then stopped by adding a stopping solution (3.65g EDTA-Na_2 and 250ml water), which the gel was left in overnight. The gel was then recorded by photography.

2.20. Statistical analysis

Each experiment was performed in duplicate for western blotting and in triplicate for all other assays. Each experiment was repeated 3-5 times on separate days. Western blots were analyzed using a two-way ANOVA followed by Bonferroni post-tests. Inositol phosphate, FLIPR calcium 4, cell migration, luciferase and receptor binding assays were analyzed using a two way ANOVA followed by a student's t-test. Statistical significance was set at $p<0.05$ with * = $p<0.05$, ** = $p<0.01$ and *** = $p< 0.001$.

2.21. Collaborator methods

The following methods were performed by collaborators at other institutions for kisspeptin antagonist studies, since facilities and materials needed for these studies were not available within the MRC Human Reproductive Sciences Unit.

2.21.1. GnRH neuron firing (Moenter Laboratory, USA)

The effects of the selected antagonist peptide 234 on kisspeptin-10 (1nM) stimulation of GnRH neuron firing (loose patch) was examined using loose patch recordings as previously described in procedures approved by the Animal Care and Use Committee of the University of Virginia (Nunemaker et al., 2002; Pielecka and Moenter, 2006; Suter et al., 2000). Positive control cells were recorded for a 10 minute stable baseline, and then treated with 1nM kisspeptin-10 for 10 minutes followed by a washout. To test for antagonist activity peptide 234 was added for 10 minutes at 1nM, 10nM or 100nM and then 1nM kisspeptin added along with 234 for 5 minutes.

2.21.2. Microdialysis monitoring of GnRH release in pubertal female rhesus monkeys (Terasawa Laboratory, USA)

The source, care, ethical approval and handling of monkeys, surgical procedures and microdialysis monitoring of GnRH were as previously described (Frost et al., 2008; Keen et al., 2008). Peptide 234 (10nM) in CNS perfusion fluid or vehicle alone were infused for 30 minutes through a semi-permeable membrane in four pubertal female rhesus monkeys in random order and the dialysate collected for GnRH assay. The infusion was preceded by a 90 minute control collection period and followed by 60 minute collection. Two of the 4 animals were examined twice in separate experiments totalling 6 experiments.

2.21.3. LH studies in intact and castrated rats (Tena-Sempere Laboratory, Spain)

The source, care, ethical approval, surgical procedures, experimental protocol and LH and testosterone assays were as previously described (Castellano et al., 2005; Navarro et al., 2005b; Navarro et al., 2004b). In intact adult males 5µl of 1nmol peptide 234 or 271 was injected icv or ip at 60 minute intervals over 180 minutes. The last injection was accompanied by an icv injection of 100pmol kisspeptin-10 in test animals. Blood samples were collected for LH and testosterone assay at 15 and 60 minutes after each injection and also at 120 minutes after the last injection. The same protocol was employed for castrated rats except the kisspeptin-10 injection was omitted.

2.21.4. LH studies in intact and castrated mice (Steiner Laboratory, USA)

The source, care, ethical approval, handling, surgical procedures, experimental protocol and LH assays were as previously described (Gottsch et al., 2004; Smith et al., 2005b). In initial experiments castrated mice were infused icv twice with doses of 1nmol, 5nmol and 15nmol peptide 234 and blood collected 30 minutes after the second infusion. This study was repeated at 5nmol and 15nmol with a single infusion and collection of blood

30 minutes later. To demonstrate that peptide 234 was specifically inhibiting kisspeptin stimulation of LH, intact male mice were infused icv with peptide 234 (100pmol) or vehicle, followed 5 minutes later by infusion of 100fmol kisspeptin-10 or vehicle alone and blood collected after 30 minutes.

2.21.5. LH pulsatility studies in ovariectomised ewes (Clarke Laboratory, Australia)

The source, care, ethical approval, handling, surgical procedures, experimental protocol and hormone assays were as previously described (Barker-Gibb et al., 1995; Iqbal et al., 2006). Blood samples were collected every 10 minutes for LH assay. From 180-240 minutes, four ovariectomised ewes were infused icv with vehicle or peptide 234 at 40µg/h after a loading dose of 10µg. Each ewe served as its own control, randomly receiving peptide 234 or vehicle on two occasions.

2.21.6. Puberty Studies in female pre-pubertal rats (Tena-Sempere Laboratory, Spain)

Peptide 234 was chronically infused to pubertal females. As general procedure for central delivery of the antagonist, pre-pubertal females (n=15/group) were implanted intradermally with osmotic minipumps (1 µl/h delivery rate X 7 days, Alzet mini-osmotic pump model no. 2001; Durect, Cupertino, CA) that were connected to icv cannulae, as previously described (Roa et al., 2008d). Antagonist concentration per mini-pump was adjusted to 10 nmol/24 µL. Pair-aged females infused with vehicle served as controls. The treatment spanned from postnatal d-30 to d-36. Along treatment, the animals were monitored for daily food intake, body weight gain and vaginal opening. On d-36, the animals were killed by decapitation, trunk blood was collected for LH and FSH assays. Ovarian and uterine tissues were also dissected out and weighed. A statistical analysis was performed as an ANOVA followed by Student-Newman-Keuls multiple range test, or Student t-test –for bar graph data.

2.21.7. LH surge activation in female rats (Tena-Sempere Laboratory, Spain)

A similar protocol of central infusion of peptide 234 was implemented in adult, cyclic female rats. The animals (n=9) were implanted with osmotic mini-pups in the morning of estrus, and infusion was continued until the following estrus. On the afternoon of proestrus, blood samples (250 μL) were obtained by jugular vein-puncture at two hour intervals, from 12:00 onwards, following previously published procedures (Roa et al., 2008a; Roa et al., 2008c). Additional blood samples were taken from each animal between 9:00 and 10:00 of the following estrus. A group of cyclic female rats (n=11) infused with vehicle served as controls.

Chapter Three

Development and characterisation of potent kisspeptin antagonists using *in vitro* and *in vivo* analyses to delineate physiological mechanisms affecting gonadotrophin secretion

3.0. Abstract

GnRH neurons are the final conduit through which the brain can control the reproductive system. GnRH neuron function is thought to be regulated by an afferent network of kisspeptin-producing neurons. Kisspeptin is released to act on its receptor (gpr-54) situated on GnRH neurons to stimulate GnRH secretion. However, the evidence supporting this hypothesis to date is indirect. Therefore kisspeptin antagonists were produced to analyse the structure-activity properties of kisspeptin-10 (kp-10) and elucidate the role of kisspeptin on gonadotropin production. Synthetic analogues were created by introducing amino acid changes to human kp-10 and were tested for their ability to bind to and activate or antagonise human gpr-54. A number of antagonists were identified and some of these were subjected to *in vivo* testing to elucidate functional roles for kisspeptin. Structure-activity studies of the analogues indicated that Asn^2, Trp^3, Phe^6, Arg^9 and Phe^{10} were important for the peptide to bind to the receptor. Two other residues, Tyr^1 and Leu^8 were found to be critical for receptor activation. In addition to deciphering functionally important residues within kp-10, these studies also identified four antagonists with the following consensus sequence for potent antagonism, X^1-N-W-N-X^5-F-G-X^8-R-F-NH_2 were X^1 = D-Ala or D-Tyr, X^5 = Gly or D-Ser and X^8 = D-Trp or D-Leu. One of these antagonists, peptide 234 was extensively tested *in vivo* where it was shown to inhibit GnRH neuron firing in mice and GnRH secretion in monkeys. This inhibition of GnRH secretion is a result of decreased pulse amplitude, and the same effect is seen on LH pulses in rodent and sheep models, confirming kisspeptin as a direct regulator of the HPG axis. However, peptide 234 had no effect on basal secretion of GnRH or LH in any species, suggesting differential mechanisms for regulation of basal and pulsatile secretion of these hormones. Peptide 234 also inhibited the post-castration rise in LH levels observed in male rats and mice, providing direct evidence that increased kisspeptin mediate the LH rise resulting from removal of steroid negative feedback.

The antagonist also ablated the ovulatory LH and FSH surges in female rats and delayed vaginal opening in peri-pubertal rats, confirming further roles for kisspeptin in positive steroid feedback on the LH/FSH surge and influencing the timing of the onset of puberty. In summary, kisspeptin antagonists have been developed and utilised to delineate the functionally important structural features of kp-10 and the role of kisspeptin in gonadotropin regulation.

3.1. Introduction

The HPG axis regulates reproduction via the modulation of GnRH production as a consequence of multiple pathways acting within the hypothalamus (Centeno et al., 2007; Millar, 2005; Porkka-Heiskanen et al., 1997). Kisspeptin via gpr-54 has been shown to be one of the most potent regulators of this system known to date with doses as low as 1fmol (icv) capable of stimulating GnRH secretion *in vivo*. Kisspeptin and gpr-54 have also been implicated to have roles in puberty onset, regulation of the pre-ovulatory LH surge and integration of other regulatory pathways that affect the axis such as steroid hormone feedback and seasonal breeding stimuli (Popa et al., 2008; Roseweir and Millar, 2009). The kisspeptin system was first discovered to be involved in the regulation of reproduction when two groups found that mutations in gpr-54 caused delayed or absent puberty with similar results evident in knockout mouse studies (de Roux et al., 2003; Seminara et al., 2003). Since then many studies have emerged examining the effect of kisspeptin on GnRH secretion and the onset of puberty, with a major focus on how kisspeptin and gpr-54 are regulated by sex steroids, metabolic cues and photoperiod. The latter factors can negatively regulate *KiSS-1* mRNA levels within the ARC throughout the female reproductive cycle (Pompolo et al., 2006; Rance, 2008; Shibata et al., 2007; Smith et al., 2007; Smith et al., 2005b). Estrogen can also positively regulate *KiSS-1* mRNA within either the ARC in sheep and primates or the AVPV in rodents at the time of the pre-ovulatory surge in LH production which initiates ovulation (Adachi et al., 2007; Estrada et al., 2006; Smith et al., 2005a). However, most of the data concerning the role of the kisspeptin system to date is indirect (such as

measurement of changes in mRNA levels or altered patterns of immunohistochemical staining).

To directly elucidate the importance of kisspeptin and gpr-54 in biological processes, the development of kisspeptin antagonists is a crucial requirement. The development of these synthetic analogues can be undertaken via two different strategies of structure-function analysis; the more logical approach is to introduce systematic residue changes (e.g. Alanine screening). This would involve each residue being systematically changed to Alanine, resulting initially in 10 synthetic peptide analogues. Although this would assess the function of each residue, this system is not very cost-effective (with these 10 peptides costing ~ £3300) and this cost would be amplified if any double-residue changes were then examined. The second approach, as used in this study, is to make more intuitive amino acid changes based on knowledge of the peptide structure of kisspeptin (Fig. 26). This involves making either conservative changes at positions of interest such as changing the side chain but not the overall charge of a residue, or changing L- amino acids to D-amino acids to assess the positioning of side chains. Finally, radical structural changes can also be made to change the charge or flexibility of the analogue. This approach was chosen for this study as it is more cost-effective and potentially more rapid than the systematic approach. Any antagonists identified would be very useful tools, to investigate whether blockade of the system directly affects puberty onset, GnRH/LH secretion, steroid feedback and the pre-ovulatory LH surge. Antagonists may also be useful medicines with potential application as novel contraceptives, potential for management of polycystic ovarian syndrome (PCOS) patients (where there is often elevation of gonadotropin levels) or for treatment of children with precocious puberty.

The results within this chapter cover the development and *in vitro* characterisation of synthetic kisspeptin-10 peptide analogues, enabling identification of a number of potent antagonists. Selected antagonists were then characterised using *in vivo* experiments. Kisspeptin antagonists caused inhibition of GnRH and LH pulses and decreased FSH

secretion in rodents, sheep and primates. An antagonist also caused a delay in pubertal maturation and abolished the pre-ovulatory LH surge in rats.

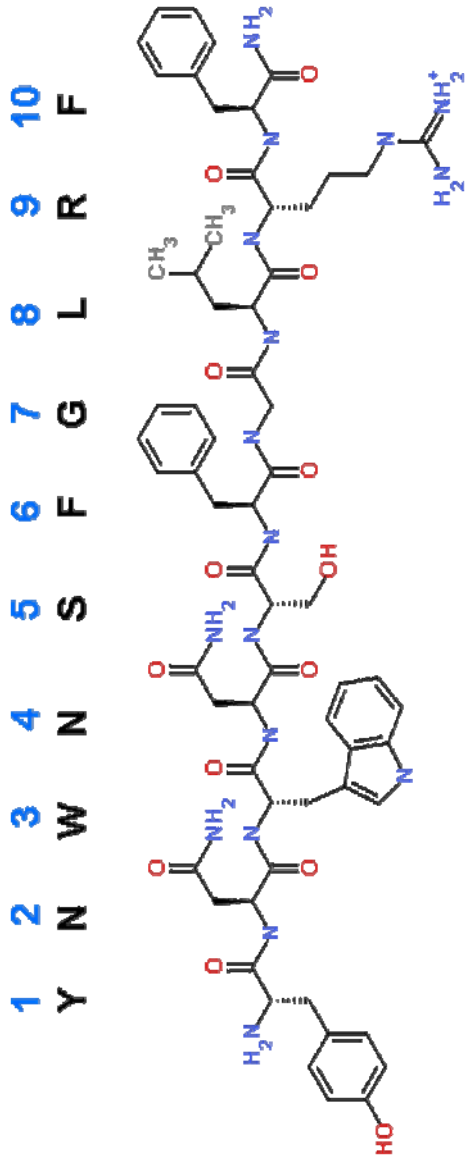

Figure 26. Schematic diagram showing kisspeptin-10 primary structure. Amino acid side chains and carbon backbone for kisspeptin-10 are indicated. Both the side chains and the carbon backbone may be important for establishing interactions with gpr-54.

3.2. Results

3.2.1. Kisspeptin analogues developed using intuitive amino acid changes bind with similar affinity to native kisspeptin-10 at human gpr-54 – Truncated peptides

Analogues were synthesised based on the structure of human kisspeptin-10 (kp-10) as this is the smallest fragment needed to bind to and activate the receptor (Muir et al., 2001; Ohtaki et al., 2001). Analogues were tested using CHO cells stably expressing human gpr-54 for their ability to bind to the receptor via a competition binding assay. A 4 hour time point was used to test the binding of analogues to human gpr-54 since initial time course studies over 5 hours, revealed this to be the time point were unlabelled kisspeptin-10 and selected analogues maximally displaced ^{125}I-labelled kisspeptin-10 (Fig. 27).

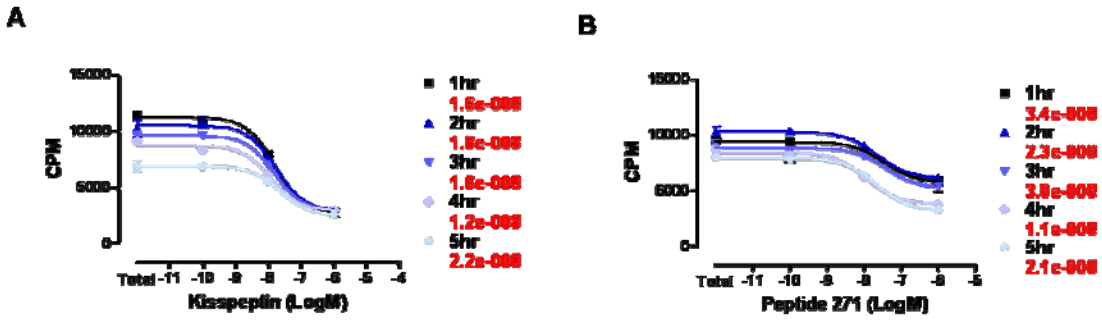

Figure 27. Time course of binding to assess maximal displacement. Time course assessing the time taken for unlabelled kisspeptin-10 or peptide 271 to maximally displaced ^{125}I-labelled kisspeptin-10 from human gpr-54. (A) Kisspeptin-10 maximally displaces labelled peptide at all time points tested. (B) Peptide 271 maximally displaces labelled peptide from 4 hours.

The results of the competition binding assays were quantified for comparison by calculating the half maximal inhibitory concentration for each analogue, known as the IC_{50}. This is the concentration of competing ligand, which displaces 50% of the specific binding of the radioligand in a competition assay and can therefore be compared

between kp-10 analogues. This IC_{50} value for an analogue is variable between assays and can therefore be converted to the absolute inhibitory constant (K_i) using the Cheng-Prusoff equation which states:

$$K_i = \frac{IC_{50}}{1 + [S]/K_m}$$

where [S] is the analogue concentration and K_m is the analogues affinity for the receptor. This gives the concentration of competing ligand which would occupy 50% of the receptors if no radioligand was present. However, since the aim of this study was to compare a large number of analogues to Kp-10 and the K_m for each analogue was not able to be calculated; only the IC_{50} was determined. The dissociation constant or K_d may also have been estimated, to give an accurate measurement of the binding affinity for each analogue. The K_d is based on the law of mass action which proposes a dynamic equilibrium exists for a reaction, e.g. between the number of unoccupied receptors/free ligand concentration and the number of occupied receptors. The K_d is the concentration of ligand at which 50% of receptors are occupied and therefore the concentration at which this equilibrium occurs. The K_d can be calculated using the law of mass action equation:

$$\text{Receptor} + \text{ligand} \underset{K_{off}}{\overset{K_{on}}{\leftrightarrows}} \text{Receptor.ligand}$$

Where K_{on} is the association rate constant and K_{off} is the dissociation rate constant. These are both proportional to the concentration of ligand and the number of unoccupied receptors. At equilibrium, the rate of association should equal the rate of dissociation giving the following equation:

$$[\text{ligand}].[\text{receptor}].K_{on} = [\text{ligand.receptor}].K_{off}$$

The K_d can then be calculated as follows:

$$K_d = \frac{K_{off}}{K_{on}} = \frac{[\text{ligand}].[\text{receptor}]}{[\text{ligand.receptor}]}$$

However, within the scope of this thesis the rate of association and dissociation where not measured, and therefore the rate constants and K_d could not be calculated. The IC_{50} value was therefore used as a measure of the apparent binding affinity for analogues.

Firstly, experiments tested whether all 10 amino acids were required for binding to the receptor using N-terminally truncated peptides possessing 5 or 7 amino acid residues. These were named peptides 188 (acFGLRF) and 186 (acNSFGLRF), respectively (Fig. 28; for continuity the amino acid residues are numbered to correspond to positions in the full length kisspeptin-10). Each of these truncated peptides had a reduced binding affinity for the receptor compared to kp-10. This is calculated as the IC_{50}, which is the concentration of peptide needed to displace the radiolabelled ligand by 50%. Peptide 188 (acFGLRF) had an IC_{50} of $1.4x10^{-7}$M for gpr-54 and peptide 186 (acNSFGLRF) had an even lower IC_{50} of $1.8x10^{-6}$M (Fig. 28 and Table 5). Therefore, it appears that binding of truncated peptides is less effective than kp-10, for which the IC_{50} is $7.7x10^{-9}$M; based on a representative graph of the mean binding curve for kp-10 (Fig. 28 and Table 5). The reduction in binding affinity seen for the 5aa and 7aa truncated analogues suggests that more than 7aa are needed to bind effectively to gpr-54. Therefore, this implies that all 10aa are needed for binding to human gpr-54 and that at least one of the N-terminal residues must be involved in this process.

Nevertheless, amino acids within the 5 amino acid truncated peptide were substituted to see if the binding affinities could be increased. These are peptides 189, 190, 191 and 200-203, and 206-207. All of these analogues had reduced affinity for human gpr-54 (Table 5); however, these analogues did highlight some residues important for binding interactions with the receptor.

As the RFamide motif at the C-terminus is hypothesised to be critical for receptor binding, we firstly investigated substitutions at these two residues. Trp^{10} was introduced into peptide 189 to assess the effects of introducing a bulkier and polar side chain into the RFamide motif. The use of Trp^{10} also introduces rigidity to the phenyl ring and

some steric hindrance thus reducing flexibility of the C-terminus. Peptide 189 (acFGLRW) had an IC_{50} of $9.5x10^{-7}$M for human gpr-54 (Fig. 29 and Table 5). The results imply that introducing a more rigid polar residue into the RFamide motif decreases the ability to bind the receptor. Next a D-Arg^{9} was substituted in place of the normal L-Arg^{9} in conjunction with the Trp^{10} substitition (peptide 200). This substitution of both residues of the RFamide motif ablated kisspeptins ability to binding to the receptor. These results confirm the importance of both the residues within this motif for receptor binding interactions (Fig. 29 and Table 5).

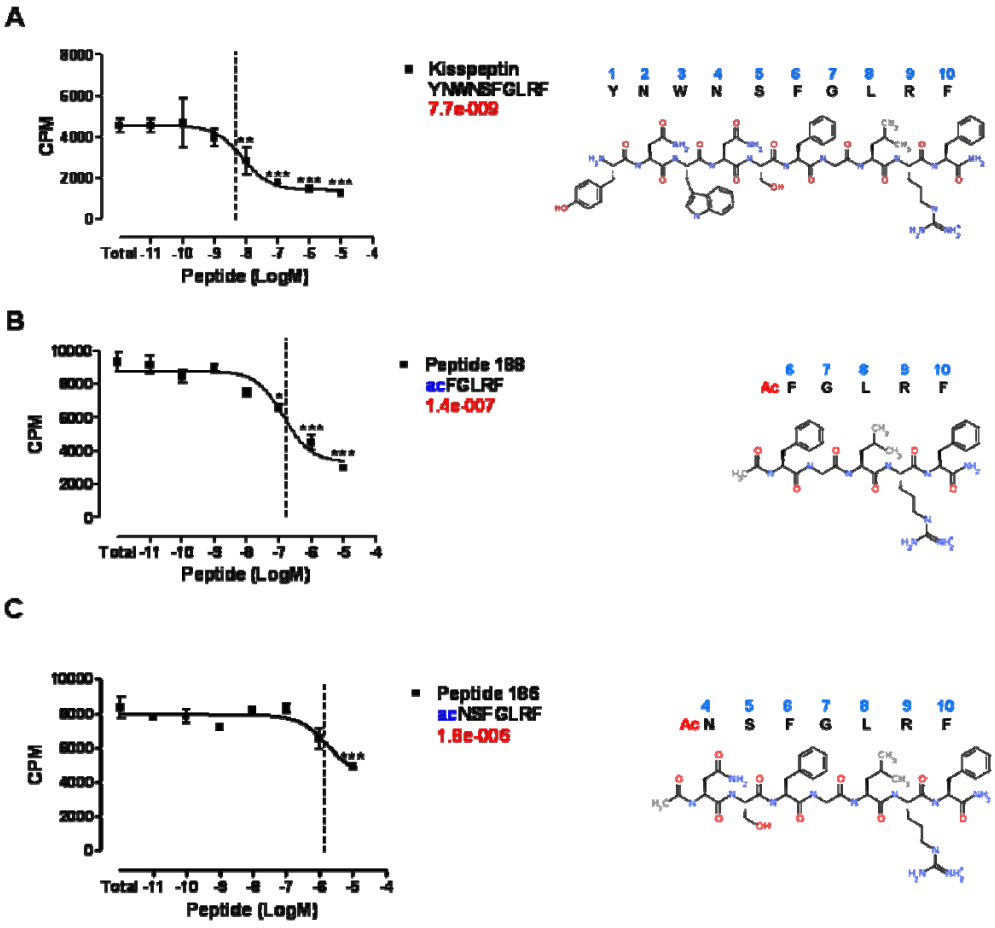

Figure 28. Truncated analogues of kp-10 have decreased binding affinity for gpr-54. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) Truncation of this peptide to five amino acids decreases the IC_{50} for gpr-54 to 140nM. (C) Increasing the length of the analogue to seven amino acids actually decreases the binding further to an IC_{50} of 1.8μM. Primary structures for each analogue are shown.

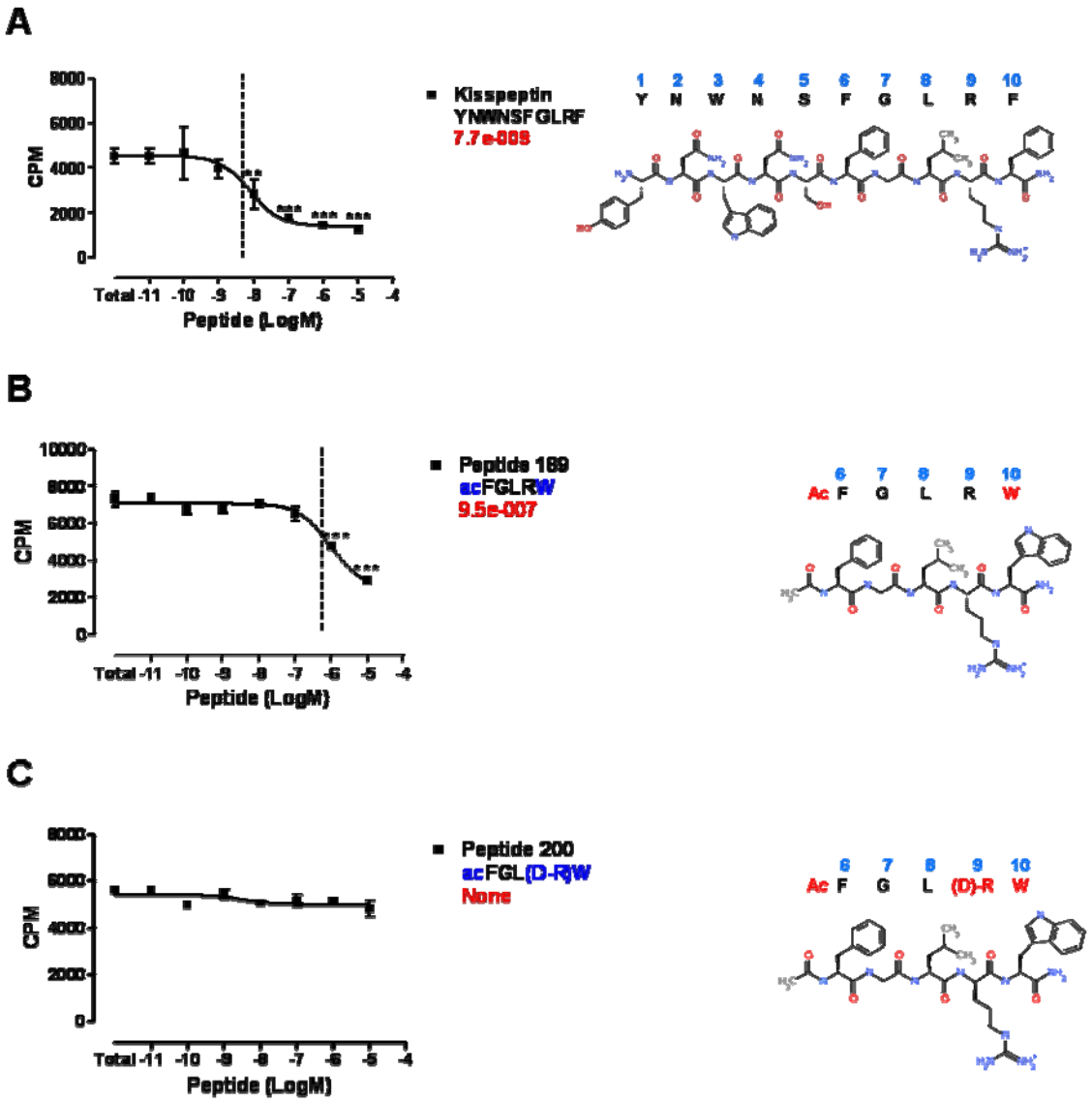

Figure 29. Substitution of both Arg^{9} and Trp^{10} of the RFamide motif in truncated analogues ablates binding. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) Substitution of Phe^{10} with Trp^{10} reduces receptor binding to an IC_{50} of 950nM. (C) Substitution of both Phe^{10} to Trp^{10} and Arg^{9} to D-Arg^{9} ablated the analogues ability to bind to human gpr-54.

Arg^{9} and Phe^{10} have been shown to be important for receptor binding in truncated peptides, and one further residue was also shown to be involved in this interaction within the N-terminus (peptides 202-203). The first residue of the truncated 5 amino acid analogue is a bulky aromatic and hydrophobic phenylalanine^{6}; this is usually within the core of the peptide structure and may evade exposure to an aqueous environment. Therefore, two substitutions were made at this position; firstly within peptide 202 an alanine is substituted for Phe^{6}. Alanine is smaller than phenylalanine and possesses a hydrophobic methyl side chain. This substitution should inform about the need for longer side chains at this position. The second substitution is a D-Phe^{6} (peptide 203) to determine if the position of the side chain in the peptide is important for binding. Peptide 202 could no longer bind the receptor (Fig. 30 and Table 5) suggesting that the side chain of phenylalanine is important for kp-10 to interact with gpr-54 along with Arg^{9} and Phe^{10}, possibly via hydrophobic interactions. Peptide 203 could also not bind to gpr-54, confirming the need for the side chain and revealing that the positioning of the side chain is important for interactions to occur (Fig. 30 and Table 5).

As none of the above truncated peptides could bind the receptor with the same or higher affinity than kp-10, this confirms that more than five residues are needed to fully bind to the receptor. Therefore further studies with truncated peptides were abandoned and full-length 10-residue analogues were developed and tested.

It was noted from studies using the truncated peptides, that neither kp-10 nor the truncated analogues could completely displace the radiolabelled ligand in the whole cell binding assay. This problem was subsequently shown to be due to the radiolabelled ligand binding to the plastic ware. Binding of ^{125}I-labelled kp-10 to the plastic ware, results in a relatively high amount of non-specific binding when kp-10 and the analogues are tested for binding to the receptor in a displacement assay. This makes it difficult to accurately determine the specific binding for some peptides due to overlap with the high non-specific binding. The best way to analyse this data would be to subtract non-specific

binding at each displacement point, however this would be difficult as the results would need to be compare to non-transfected cells plated at the same density.

The high non-specific binding seen for some analogues may also be due to some peptides aggregating or forming secondary or tertiary structural lattices, since this would impair the ligand binding interactions giving a false level of non-specific binding. To try to combat these effects a variety of solvents such as weak acids, DMSO, detergents and distilled water were utilised to try to inhibit formation of secondary structures, but this approach had limited success. The temperature of analogue stock solutions was also varied to try to break down any hydrogen bonding within potential lattices but this also failed to cause a significant effect. These results suggest that lattices are not being formed in these analogues but this could to be investigated using light scattering spectroscopic techniques (e.g. infrared spectroscopy or circular dichroism). In this assay, any peptides undergoing aggregation would cause a higher amount of light scattering than disaggregated peptides.

Other factors may also affect the assay conditions for ligand binding interactions such a purity and solubility which might reduce the effective ligand concentration and specific receptor activity and receptor dimerisation when over-expressed. It is important to know that the ligand solubility exceeds the concentration of the peptide under investigation. The concentration is determined using the molecular weight and weight of peptide per millilitre (mg/ml), which can then be used to calculate the molarity (M). If the peptide does not fully dissolve then the mg/ml will be incorrect giving an inaccurate calculation of the concentration. Another problem related to concentration determination is the purity of the peptide, the lower the purity of the analogue. This may lead to an overestimation of the concentration of the peptide solution which leads to an underestimation of the binding affinity for the peptide. The amount of peptide per millilitre and therefore the concentration may therefore be confirmed in several ways such as using a nanodrop spectrometer to determine the absorbance at 280nm if the peptide contains tryptohans or tyrosines. Based on the known extinction coefficients

and the number of the two absorbing amino acids, the molar concentration can be determined with much better accuracy compared to weighing in a sample of low mg amounts. HPLC chromatography using absorbance or fluorescence detection combines the concentration determination with the measurement of the sample purity. As for receptor dimerisation, this requires specialised fluorescence resonance-energy transfer (FRET) techniques using tagged receptors or immunoprecipitation with specific antibodies to determine if any receptor dimerisation actually occurs. However, as tagged receptors are not yet available and no reliable specific antibodies have been made, it is not known whether gpr-54 forms dimers. However, as only IC_{50} were calculated within this thesis, as assay condition dependent relative estimation of affinity, receptor dimerisation has not been taken into account accounted for in these studies.

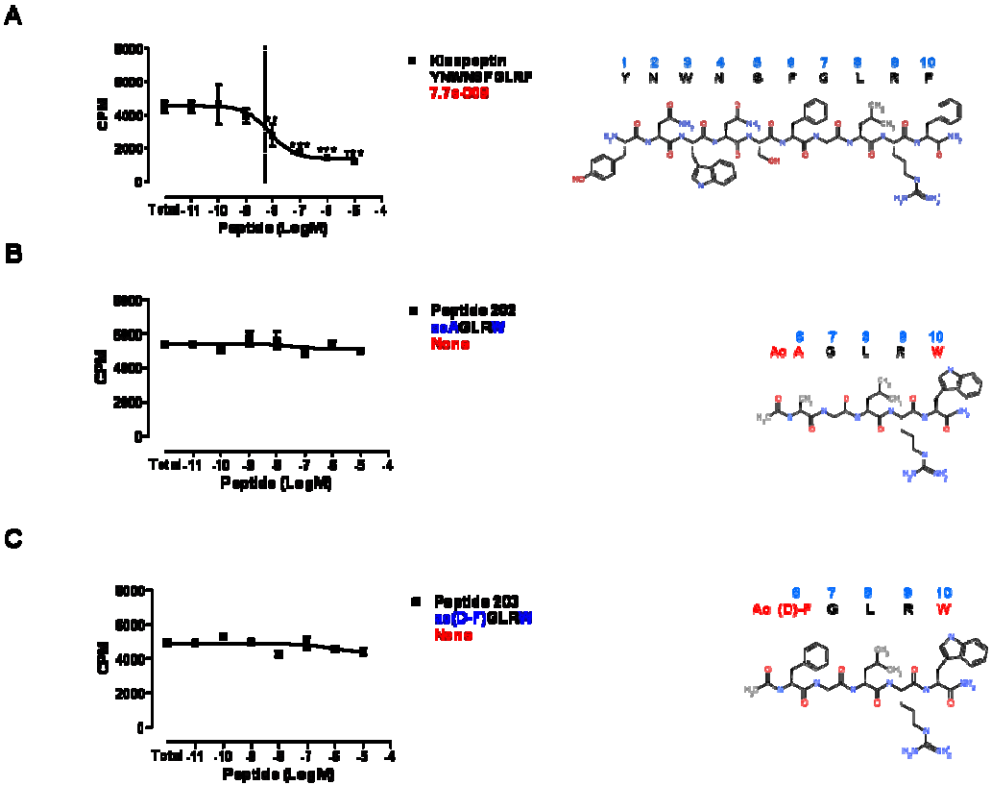

Figure 30. Position 6 amino acid changes are not tolerated. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) Substitution of Phe^6 with Ala^6 abolishes receptor binding due to the absence of the aromatic side chain. (C) D-Phe^6 is also not tolerated confirming the side chain properties.

3.2.2. Kisspeptin analogues developed via intuitive amino acid changes bind with similar affinity to native kisspeptin-10 at human gpr-54 – Full length peptides

For the first full-length substitutions the six most C-terminal residues were substituted, as it had been hypothesised that these residues were involved in binding to the receptor due to the high level of conservation of these residues between vertebrate species and the RFamide motif within this family; peptides 208-213 and 288 (Table 4).

Peptide	Species	Sequence
FMRFamide	Clam	F M R F NH2
LPLRFamide	Chicken	L P L R F NH2
GnIH	Quail	S I K P S A Y L P L R F NH2
NPFF	Human	F L F Q P Q R F NH2
NPAF	Human	A G E G L S S P F W S L A A P Q R F NH2
PrRP20	Human	T P D I N P A W Y A S P G I R P V G R F NH2
RFRP-1	Bovine	S L T F E E V K D W A P K I K M N K P W N K M P P S A A N L P L R F NH2
RFRP-3	Bovine	A M A H L P L R L G K N R E D S L S R W V P N L P Q R F NH2
Kisspeptin-1	Human	G T S L S P P P E S S G S P Q Q P G L S A P H S R Q I P A P Q G A L V Q R E K D L P N Y N W N S F G L R F NH2
mammalian	Bovine	G A A L C P P E S S A G P Q R L G P C A P R S R L I P S P R G A V L V Q R E K D V S A Y N W N S F G L R Y NH2
	Rat	R T S P C P P V E N P T G H Q R P P C A T R S R L I P A P R G S V L V Q R E K D M S A Y N W N S F G L R Y NH2
	Mouse	R S S P C P P V E G P A G R Q R P L C A S R S E L I P A P R G A V L V Q R E K D L S T Y N W N S F G L R Y NH2
	Opossum	L A M L C P S D E A S D P L W P G L C P T R S R L I T A P Q G A L L V E R E K D M S T Y N W N S F G L R Y NH2
	Xenopus	L L C R R K K S L S T G H P W S T D S L L P S R S I S A P E G E F L V Q R E K D L S T Y N W N S F G L R Y NH2
fish	Zebrafish	P T D G S P P S K L S A L F S M G A G P Q K N T W W W S P E S P Y T K R R Q N V A Y Y N L N S F G L R Y NH2
	Medaka	K E W P K D R S S D G G T P M V G C W M V K A L H P V A I K K R Q D L S S Y N L N S F G L R Y NH2
	Lamphrey	Y D F P G S G G S V D R A F M S P L H F Y P M L R A R M R S L P A S D A D E K K G S T Y N L N S F G L R F NH2
Kisspeptin-2	Zebrafish	M E R R Q F E E P S A S D D A S L C F F I Q E K D E T S Q I S C K H R L A R S K F N Y N P F G L R F NH2
fish	Medaka	I L R R S E D D S A A G G A G L C S S L R E D D E Q L L C A D R R S K F N Y N P F G L R F NH2
	Lamphrey	V C R H A A E T P R L L R L R A L R G G H D L D A G L T D G E A L P R S A E Q D V T E F N Y N P F G L R F NH2

Table 4. Primary sequences for RFamides including kisspeptin. Primary sequences for RFamides showing species where discovered. Sequences are also shown for kisspeptin-1/2 in a variety of mammalian and fish species. Boxes indicate conserved residues.

In order to increase the flexibility of the peptide Ser^5 was substituted with the achiral, glycine in peptide 208. Due to the small hydrogen atom in place of a side chain, this allows glycine to create flexibility within the peptide, to promote conformational changes. This substitution retained binding to the receptor with an IC_{50} of $1.2x10^{-7}$M (Fig. 31 and Table 5). As this replacement was well-tolerated by the peptide, it was

included in the majority of subsequent analogues. Next, amino acid changes at Leu^8 were attempted (Fig. 32). Leu^8 is a hydrophobic and aliphatic residue found in the peptide core, likely to be involved in interactions with other hydrophobic residues. Peptide 209 had a D-Leu^8 substitution, to check if the specific location of side chains was important for binding. Peptide 210 and 228 had D-Trp^8 in place of Leu^8 to test the effect of steric hindrance due to a bulky side chain within the C-terminal region upon binding. However, as this is combined with Gly^5 this will still allow flexibility within the central region. Peptide 228 also had an acetyl group added to the N-terminus to try to extend the half-life of the peptide when exposed to amino-peptidases in a biological environment. Peptide 209 had decreased ability to bind the receptor, with an IC_{50} of $2.8x10^{-6}$M, but exhibited similar capacity to displace radiolabelled ligand compared to kp-10. Peptide 210 was, however, able to bind the receptor with an IC_{50} close to that of kp-10 at $6x10^{-9}$M. The results were similar for peptide 228 although the IC_{50} was higher at $1x10^{-11}$M (Fig. 32 and Table 5). Therefore, it appears that changes within residue 8 that increase steric hindrance do not significantly affect the binding properties of kp-10. However, changing the steric position of the Leu^8 side chain is not tolerated. Also although displacement of radiolabelled ligand seems lower all the analogues (Peptides 208, 209 and 228) displace to a similar percentage as kp-10, the higher non-specific background is probably due to increase binding to plastic ware when total counts are increased due to a higher cell number (Fig. 32).

The contribution of Phe^6 was next investigated in the full-length analogues since changes to this residue had ablated binding in the truncated peptides. Two substitutions were made at this position in conjunction with Gly^5, firstly in peptide 211, this residue was replaced with D-Phe^6 as in the truncated peptides the steric orientation of the large side chain appeared important for receptor binding. The second change incorporated a D-Trp^6 residue in peptide 212, again to test for the effects of steric hindrance but as this residue is close to Gly^5 this may also affect the flexibility of the central region. All of the above changes to Phe^6 caused a significant shift in binding affinity to IC_{50} values around 1μM (~10^{-6}M). This suggests that Phe^6 is critical for receptor binding via interactions

between its side chain and gpr-54, since even changing the position of the side chain ablates binding (peptide 211, Fig. 33 and Table 5).

The above results examining the effects of substitutions of C-terminal residues suggest that binding involves Phe^{6}, Arg^{9} and Phe^{10}, this is in line with data published after this work was completed describing the pharmacophore created by these residues for binding to gpr-54 (Orsini et al., 2007).

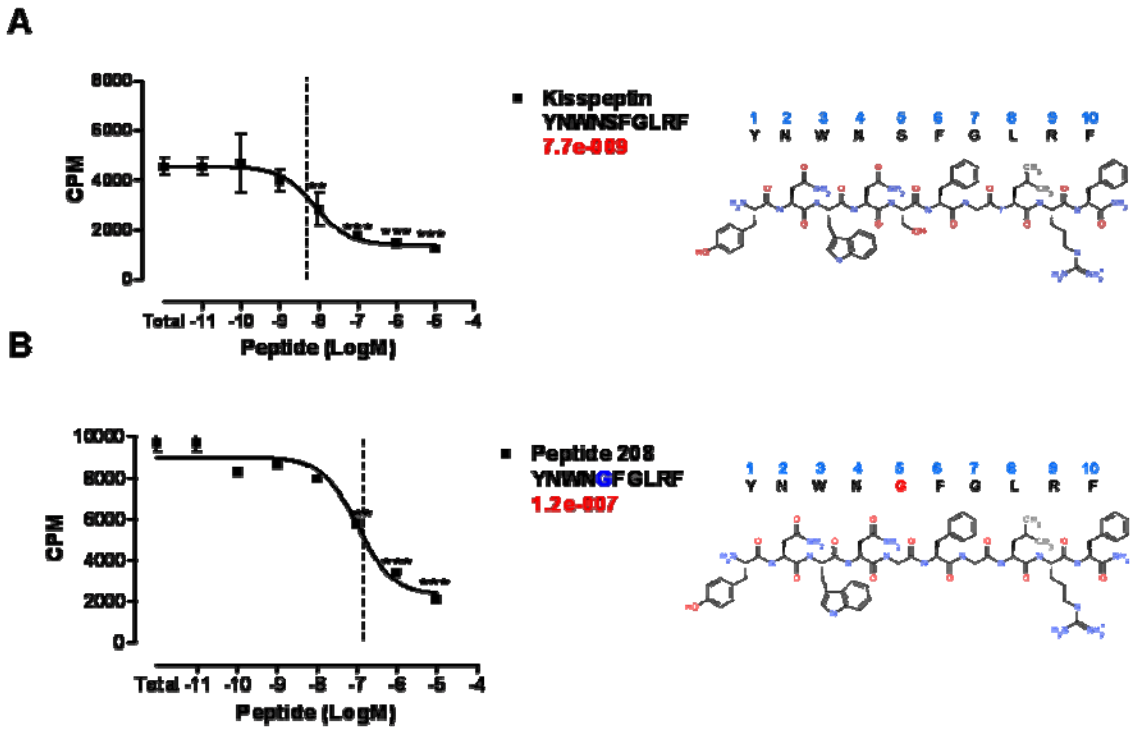

Figure 31. Glycine substitution at Ser^{5} retains binding. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) Peptide 208, with a Glycine at position 5 binds the receptor with an $IC_{50}$of 120nM and effectively displaces the labelled ligand by 75% at 10µM. Amino acid structure is also shown.

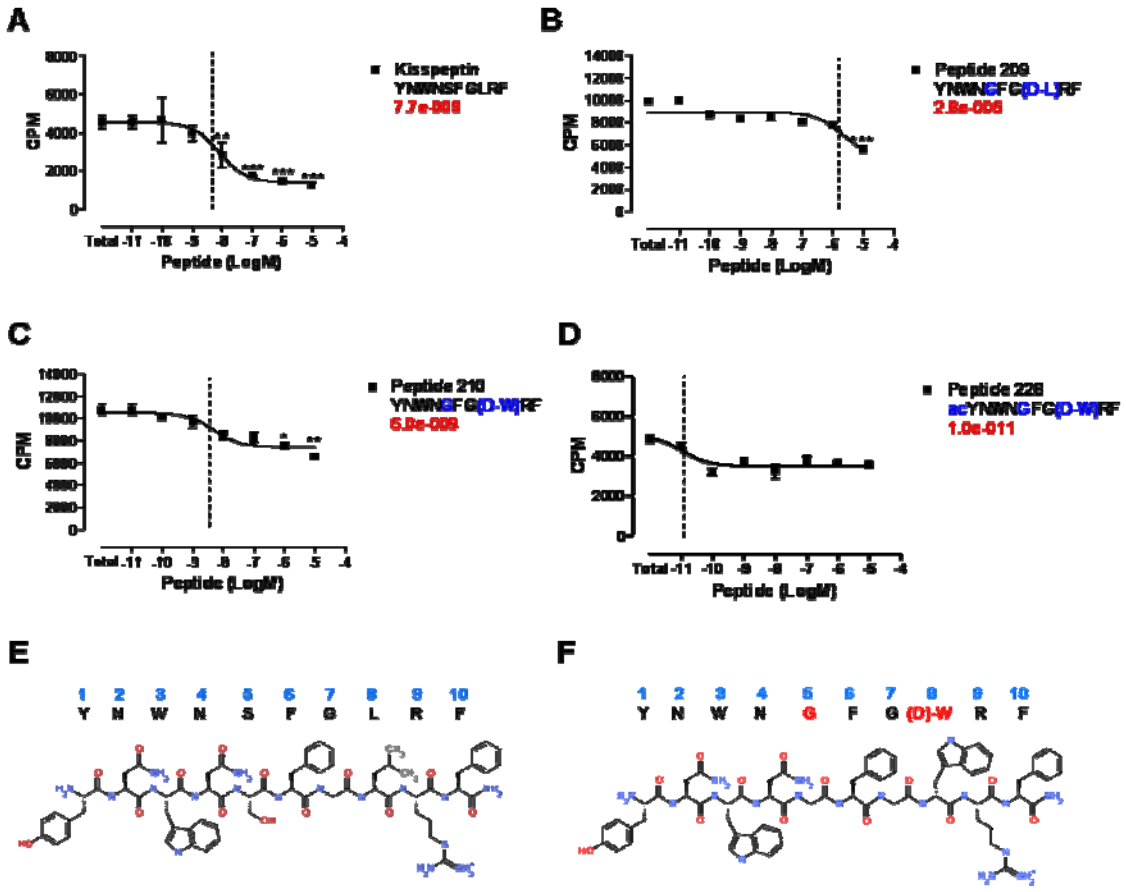

Figure 32. Amino acid changes at Leu^8 retain receptor binding. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) D-Leu^8 decreases binding affinity, with an IC_{50} value of 2.8μM. (C) D-Trp^8 retains a binding affinity an IC_{50} of 6nM similar to kp-10. (D) Acetylation of peptide 210 increases affinity, but decreases displacement. (E, F) Amino acid structures of kisspeptin-10 and peptide 210.

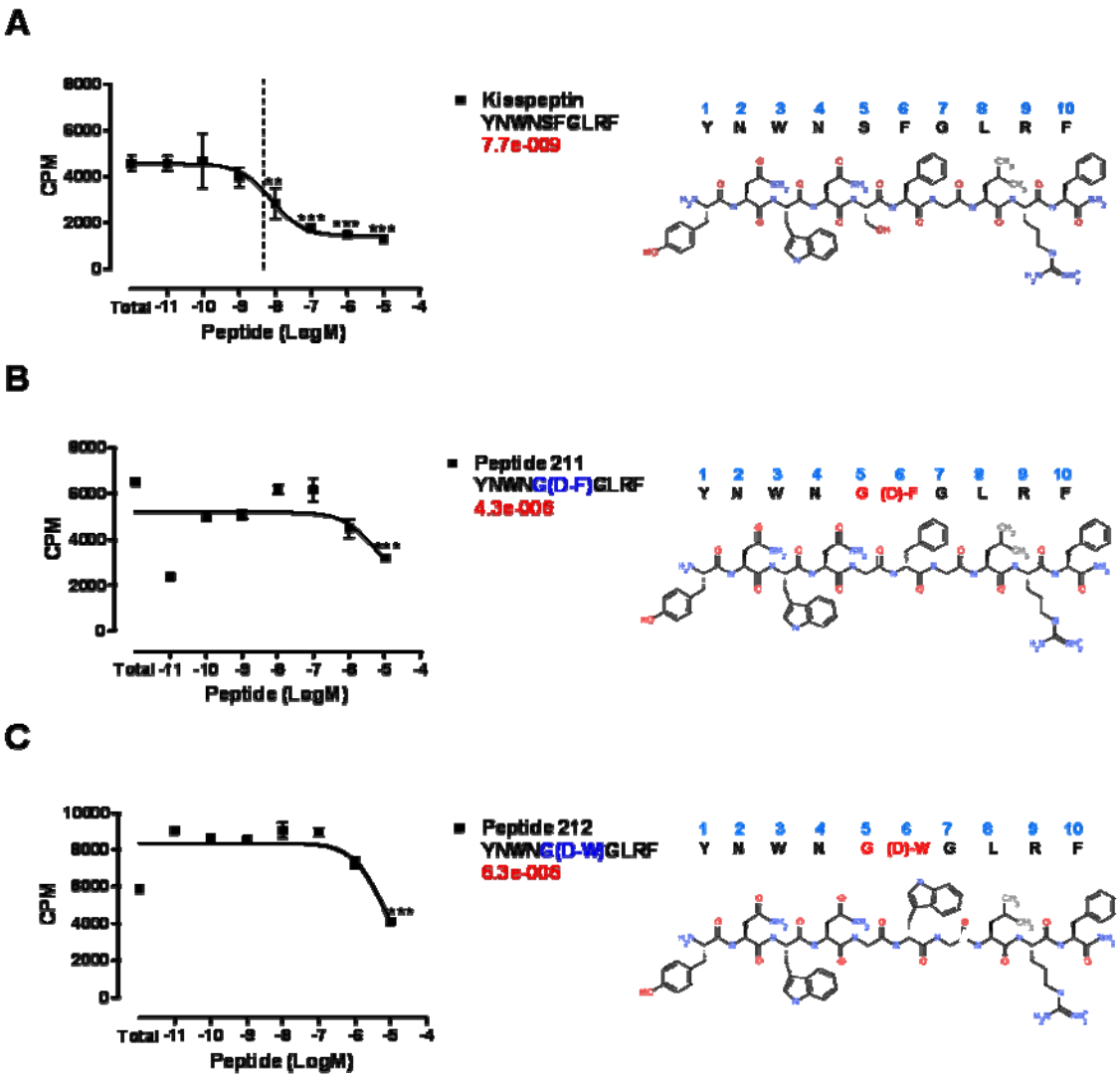

Figure 33. Phe^6 is critical for receptor binding. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) Replacement of Phe^6 with D-Phe^6 significantly reduces the IC_{50} to 4.3μM. (C) D-Trp^6 also causes a similar reduction in binding to an IC_{50} value of 6.3μM.

Following demonstration that the C-terminal residues of kisspeptin are involved in binding to receptor, attentions were turned to the five N-terminal residues to examine if receptor binding also involved N-terminal interactions. As peptide 210 could bind with a similar affinity to kp-10 (Fig. 32), the D-Trp^{8} substitution was incorporated into all further analogues in conjunction with glycine at position five. However, as Phe^{5} has been demonstrated to tolerate a glycine substitution to promote flexibility, the next changes examined if other residues would also be acceptable. Three more substitutions were tried; these were D-Trp^{5} (Peptide 229); D-Ala^{5} (peptide 233) and D-Ser^{5} (Peptide 273). D-Trp^{5} was used for the opposite reasons to glycine, to hinder conformational changes rather than increase flexibility and the D-Ala^{5} and D-Ser^{5} substitutions may cancel any interactions made at this position, due to a decrease in the side chain length. Peptide 229 was unable to bind to gpr-54, suggesting flexibility is needed in this region. Peptide 233 and 273 could still bind with an IC_{50} value of $7.9x10^{-9}$M and $1.4x10^{-10}$M, respectively, but again the displacement of radiolabelled ligand is lower (Fig. 34 and Table 5) implying that glycine at this position allows the greatest amount of receptor binding and that side chain interactions are not important within this residue. Therefore, flexibility is more important at this position than side chain interactions.

Substitutions were then made for the three most N-terminal residues in combination with Gly^{5} and D-Trp^{8}; however Asn^{4} was not substituted in these studies. Two changes were made at Tyr^{1}; firstly this residue was replaced with D-Tyr^{1} (Peptide 230) to test side chain positioning and then with D-Ala^{1} (Peptide 234) to test the importance of side chain interactions for binding. Peptide 230 was still able to bind to the receptor but the displacement of radiolabelled ligand was decreased, whereas peptide 234 could bind the receptor with similar binding affinity to kp-10 and exhibited a greater amount of displacement compared to peptide 230 (Fig. 35 and Table 5). Therefore, we can assume that Tyr^{1} is not important for binding to the receptor. Two similar substitutions were made in place of Asn^{2}. Peptide 232 incorporated a D-Asn^{2} and peptide 236 had a D-Ala^{2} in place of the asparagine removing the basic charge at this position. Peptide 232 and peptide 236 could not bind to the receptor (Fig. 36 and Table 5). These results imply that

Asn^2 interacts with the receptor to assist binding probably via hydrogen bonding and that the presence and positioning of the side chain is critical to this interaction. The results also suggest that a basic residue is needed at this position for receptor binding to take place. Finally, the same substitutions were made for Trp^3 giving peptide 231 with D-Trp^3 and peptide 235 with D-Ala^3. Peptide 231 and peptide 235 could not bind to gpr-54 (Fig. 37 and Table 5). This suggests that Trp^3 may also form interactions with the receptor and that the presence and positioning of a side chain is again important for this interaction and binding to occur.

These results from examining the effects of residue substitutions within full-length kisspeptin-10 suggest that the main binding residues lie within the C-terminal domain via Phe^6, Arg^9 and Phe^{10}, as was proposed within truncated analogues, and within the N-terminal domain via Asn^2 and Trp^3. This would account for the reduced binding in truncated peptides where Asn^2 and Trp^3 were removed. The study of the full-length analogues also suggests that the functional peptide conformation requires flexibility in the centre of the peptide as bulky residues at position 5 ablate receptor binding. The introduction of rigidity via D-Trp^8 within the C-terminus is tolerated well suggesting that this residue does not contribute to receptor binding interactions. Similar results were seen with Tyr^1 substitutions, again suggesting this residue has no involvemet in receptor binding.

Although further analogues were made, these were used to investigate receptor activation and to screen for kisspeptin antagonists, since most residues within kp-10 have been tested for effects on binding to human gpr-54 in the above research.

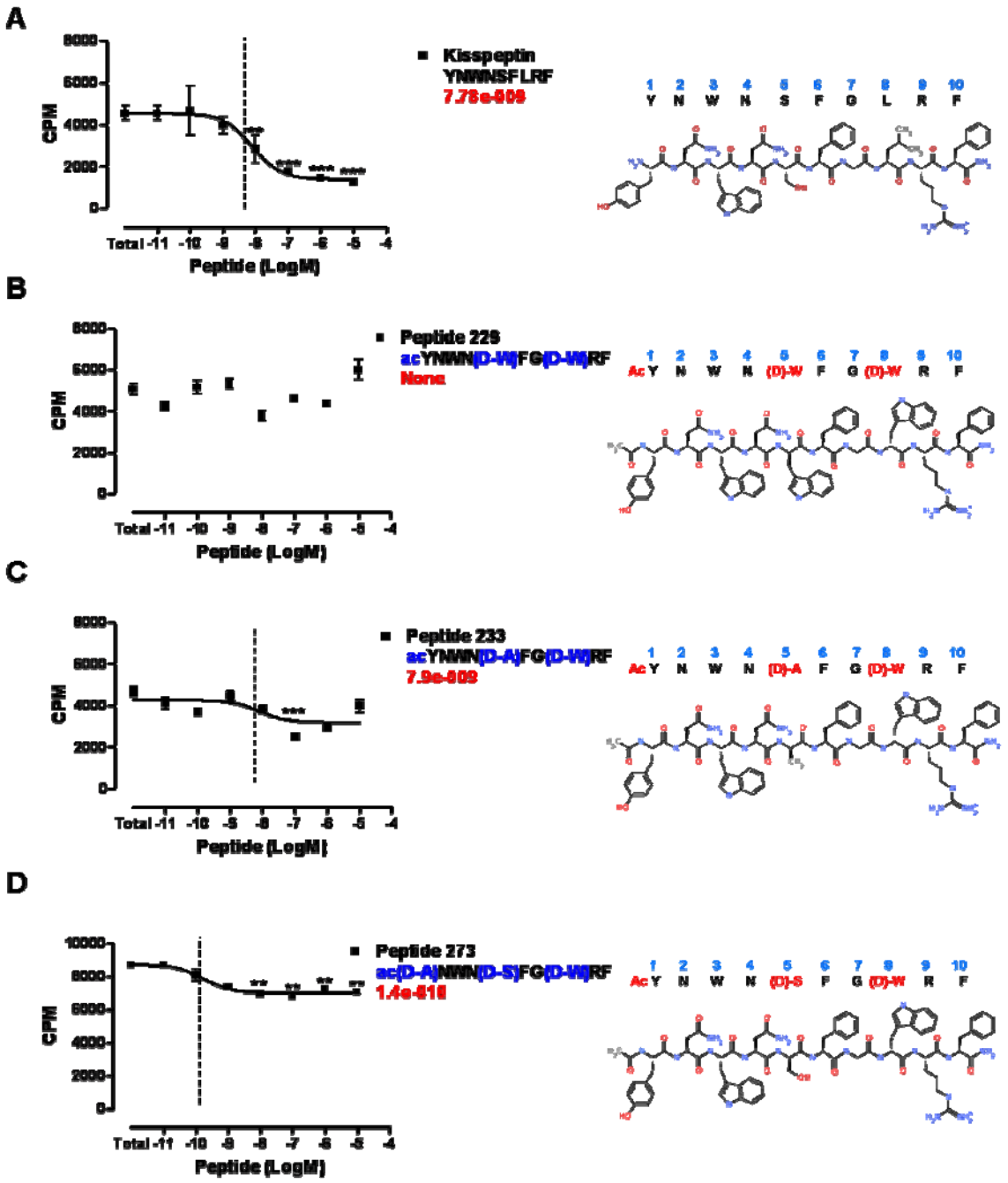

Figure 34. Amino acid changes at Ser^5 need flexibility. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) D-Trp at position 5 abolishes binding. (C) D-Ala^5 retains binding with an IC_{50} of 7.9nM as does (D) D-Ser^5 with an IC_{50} of 140pM. Amino acid composition is also shown for each analogue.

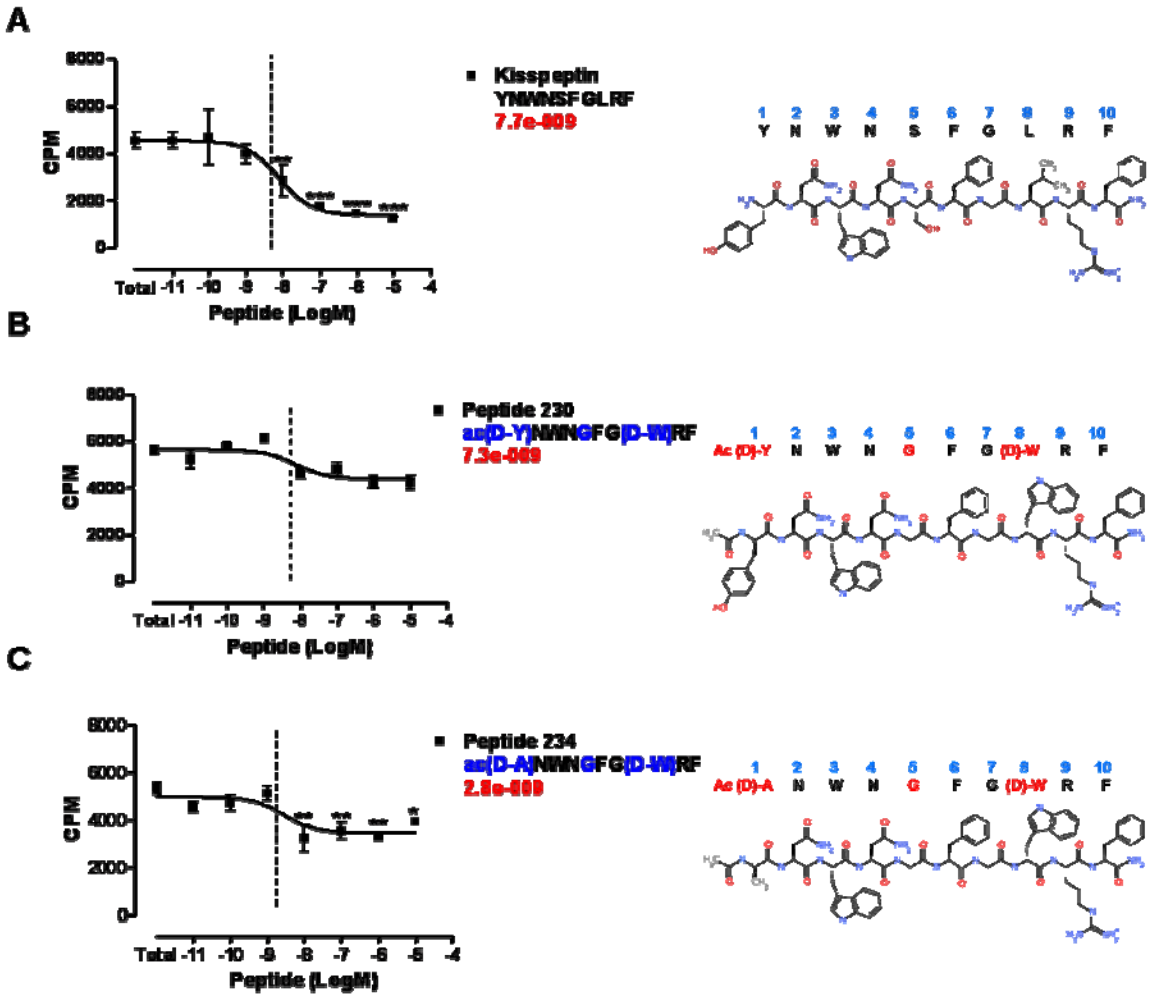

Figure 35. Tyr[1] is not involved in receptor binding. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) D-Tyr at position 1 retains ability to bind to the receptor with an IC_{50} value of 7.3nM. (C) Peptide 234 with a D-Ala[1] binds with a similar IC_{50} value to kp-10 of 2.8nM. Amino acid structure for each analogue is also shown.

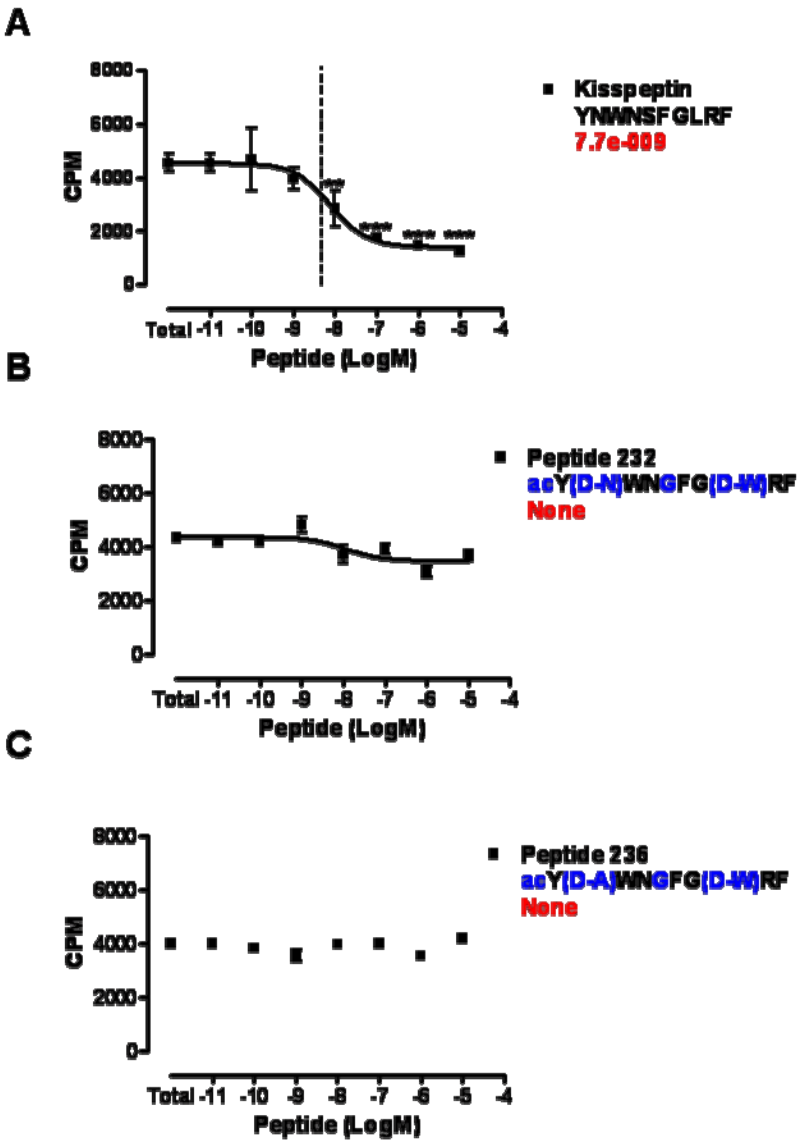

Figure 36. Asn[2] amino acid substitutions affect binding. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) D-Asn at position 2 cannot bind the receptor. (C) Similarly, D-Ala at position 2 also ablates receptor binding.

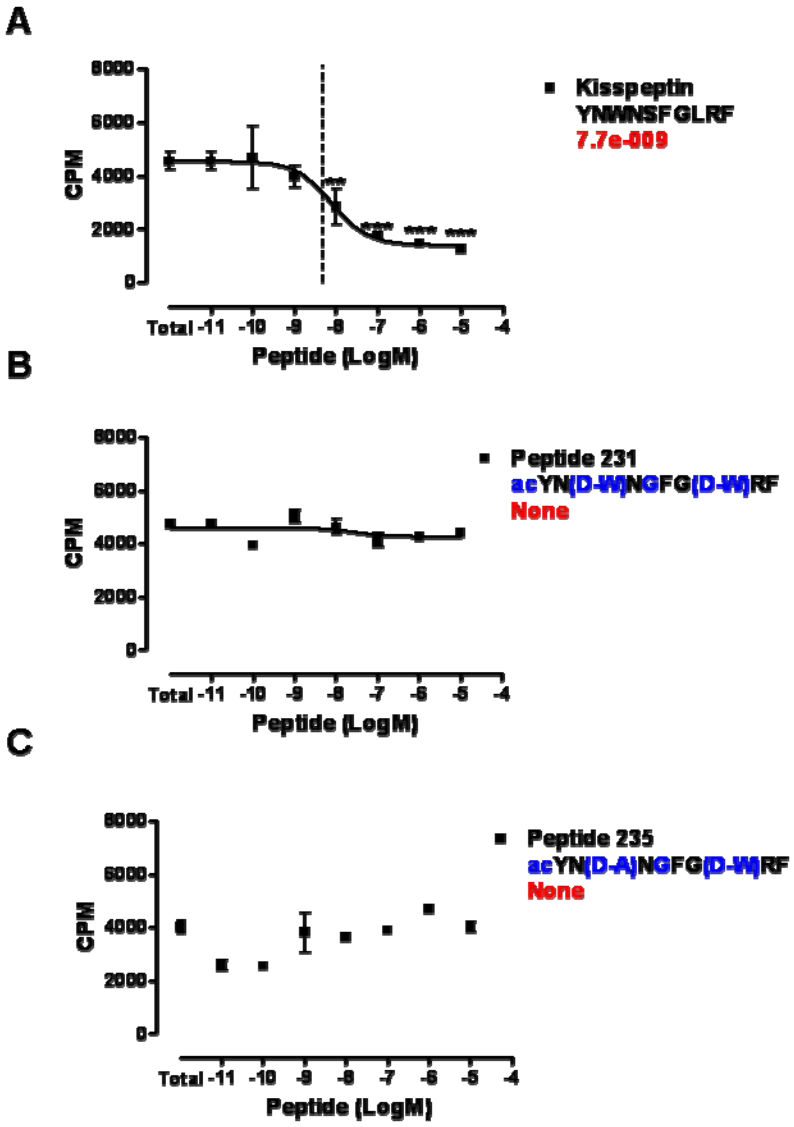

Figure 37. Trp^3 interacts with the receptor. (A) Kp-10 binds strongly to gpr-54 with an IC_{50} of 7.7nM. (B) D-Trp^3 at this position cannot bind to the receptor and (C) substitution with D-Ala^3 is not tolerated and binding is abolished due to lack of side chain.

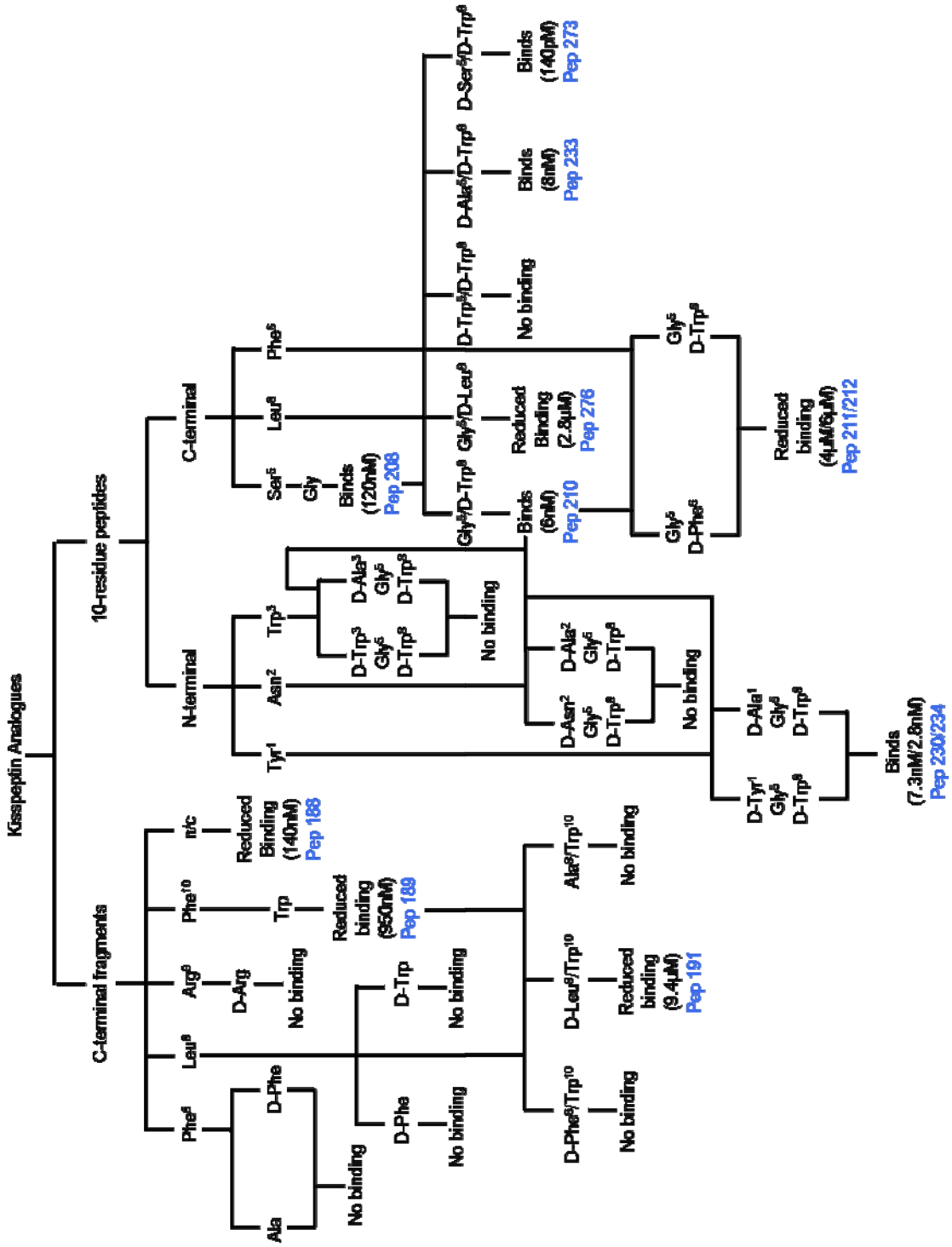

Table 5. Diagram of amino acid substitution effects on receptor binding. Flowchart for amino acid substitutions within truncated and full-length peptide analogues showing the effects of each substitution on binding. The IC_{50} for analogues that exhibit binding is shown in brackets and peptide number in blue.

3.2.3. Some kisspeptin analogues inhibit kisspeptin-10 induced inositol phosphate production and intracellular calcium release

After testing for receptor binding, analogues were tested for receptor activation via phospholipase C-mediated inositol phosphate (IP) production and antagonistic properties via inhibition of kisspeptin-stimulated inositol phosphate production. An antagonist should bind to the receptor but not activate it and ideally should inhibit kisspeptin-stimulated IP production by >80%. Kisspeptin activates the receptor with a mean EC_{50} of $3.9x10^{-9}$M; therefore a dose of 10nM was used for antagonist studies. For logistical reasons, experiments for each analogue were not always performed on the same day and although cells were routinely plated at $1x10^{-5}$ cells/well, variation in lengths of cell culturing causing variation in cell numbers can account for inter-experimental differences in basal and kp-10-induced levels of IP. Large variations were seen with some analogues and this may be due to the need to handle multiple plates per analogue for each experiment.

Effects on inositol phosphate production

Truncated peptides were tested first even though they had little receptor binding. All of these analogues could activate the receptor except peptides 201 and 206-7, which all had substitutions of Leu^8. Truncating the receptor to 5 amino acids retained its agonistic properties (peptide 188-9). Changes to position 6 (peptide 202-3) had no antagonistic effects and changes to position 9/10 (peptide 200) could not bind, activate or antagonise the receptor. However, position 8 changes did not intrinsically activate IP production and had weak antagonistic properties. Peptide 201 possessing Ala^8 could antagonise the kp-10 stimulation by 67% with an IC_{50} of $7x10^{-9}$M, peptide 206 with a $D\text{-}Trp^8$ could also antagonise the kp-10 stimulation by 71% with an IC_{50} of $5x10^{-9}$M but peptide 207 with a $D\text{-}Phe^8$ could only antagonise by 53% with an IC_{50} of $3x10^{-9}$M (Fig. 38 and Table 6). These results suggest that Leu^8 is important for activation of gpr-54 and that large residues are the most effective residues for antagonism. However, as none of these truncated kisspeptin-10 analogues could antagonise by more than 70% they were not

tested further and attentions were turned to the full-length analogues. Also the dose response curves where peculiar with low doses sometimes more effective than higher doses in some cases due to some agonist activity.

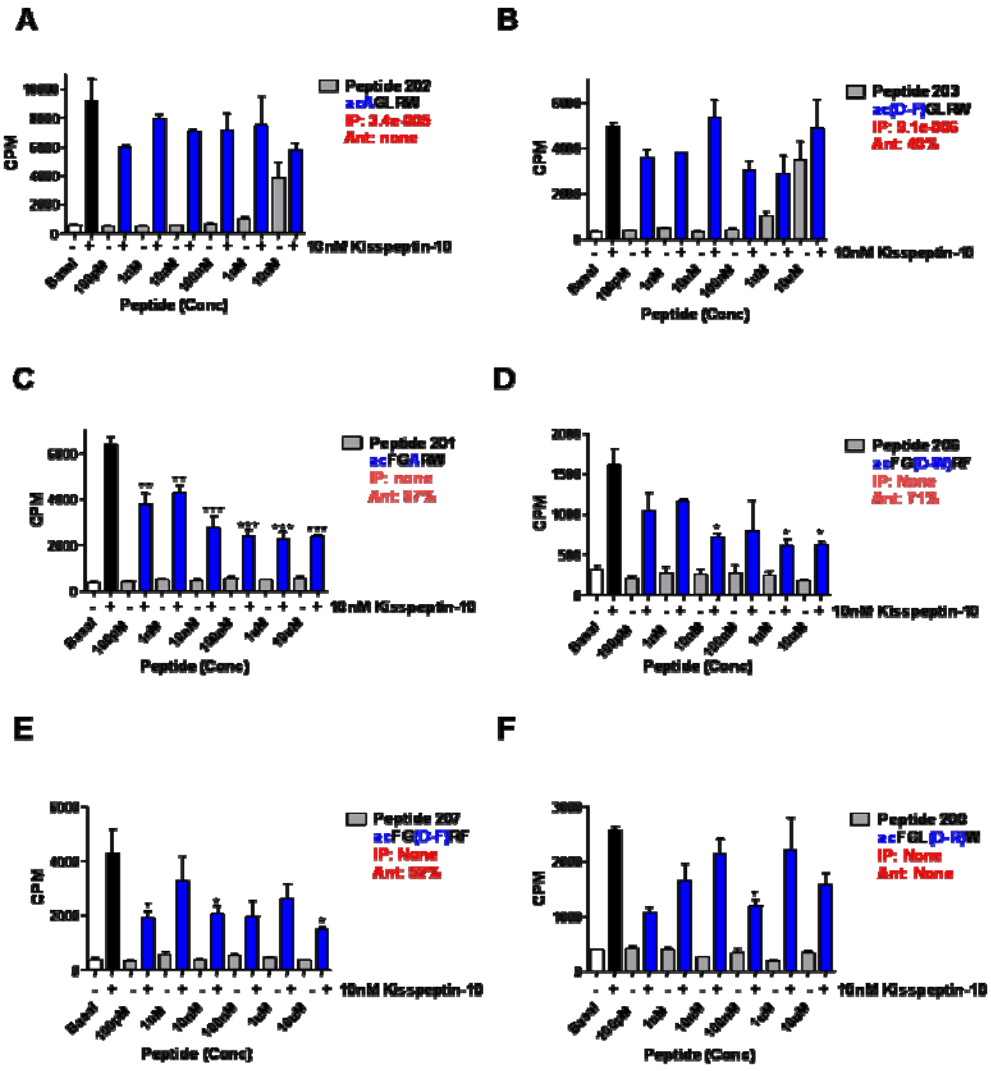

Figure 38. Truncated peptides with changes at position 8 create antagonism. Graphs showing both intrinsic IP release (light grey) and antagonism of kp-10 stimulated IP release (dark grey). IP is the concentration at which stimulation reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) Ala^6 can stimulate IP release and cannot antagonise gpr-54. (B) D-Phe^6 also stimulates IP but can antagonise by 40%. (C) Ala^8 does not produce an intrinsic IP response and antagonises by 67% with an IC_{50} of 7nM. (D) D-Trp^8 further antagonises to 71% with an IC_{50} of 5nM. (E) D-Phe^8 is less effective and only antagonises by 52%. (F) Amino acid changes to Arg^9 disrupt binding and activation of the receptor with little antagonism.

Kisspeptin Analogues (CHO cells)												Dose Response		Antagonistic IP Inhibition		Possible
No.	Peptide Sequence											Bind(IC50)	IP(EC50)	IC50	% inh max	Antagonists
Kiss	Y	N	W	N	S	F	G	L	R	F	NH_2	7.77E-09	3.90E-09	n/a	n/a	n/a
186			ac	N	S	F	G	L	R	F	NH_2	1.84E-06	5.90E-07	n/a	agonist	poor
187	Y	N	W	N	S	F	G	L	R	W	NH_2	1.50E-07	4.00E-08	n/a	36%	poor
188					ac	F	G	L	R	F	NH_2	1.43E-07	8.70E-08	n/a	agonist	poor
189					ac	F	G	L	R	W	NH_2	9.46E-07	3.60E-08	n/a	agonist	poor
190					ac	F	G	(D)-F	R	W	NH_2	No displacement	2.00E-06	n/a	agonist	poor
191					ac	F	G	(D)-L	R	W	NH_2	9.39E-06	1.80E-06	n/a	agonist	poor
200					ac	F	G	L	(D)-R	W	NH_2	No displacement	No IP	n/a	0%	none
201					ac	F	G	A	R	W	NH_2	4.02E-03	No IP	7.00E-09	67%	poor
202					ac	A	G	L	R	W	NH_2	No displacement	3.40E-05	n/a	0%	none
203					ac	(D)-F	G	L	R	W	NH_2	No displacement	9.10E-06	n/a	40%	poor
206					ac	F	G	(D)-W	R	F	NH_2	No displacement	No IP	5.00E-09	71%	good
207					ac	F	G	(D)-F	R	F	NH_2	No displacement	No IP	3.00E-09	52%	poor

Table 6. Truncated kisspeptin-10 analogues. Table showing IC_{50} values for binding and antagonism, and EC_{50} values for intrinsic IP release. The percentage of maximal inhibition at micromolar concentration are given along with an indication for possible antagonists. Blue indicates an analogue with antagonistic potential and No displacement = no displacement of kp-10.

Amino acid changes within the C-terminal region were focused on, to determine residues within this region involved in receptor activation or creation of antagonism. Ser^5 to Gly^5 substitution in kp-10 (peptide 208) inhibited kp-10 stimulation of IP by 54% with an IC_{50} of $1x10^{-8}$M (Fig. 39 and Table 7). Therefore, this change was incorporated into subsequent analogues. However, as this analogue could still stimulate an intrinsic IP release with an EC_{50} of $4.5x10^{-8}$M it implies that this substitution alone is insufficient for antagonism. Analogues comprising Gly^5 and substitutions at Phe^6 exhibited some antagonism but could still activate the receptor at high concentrations. Peptide 211, containing D-Phe^6 and, peptide 212 containing D-Trp^6 could not antagonise any further than peptide 208 (Fig. 39 and Table 7). This implies that a Phe^6 substitution is not useful for antagonist activity.

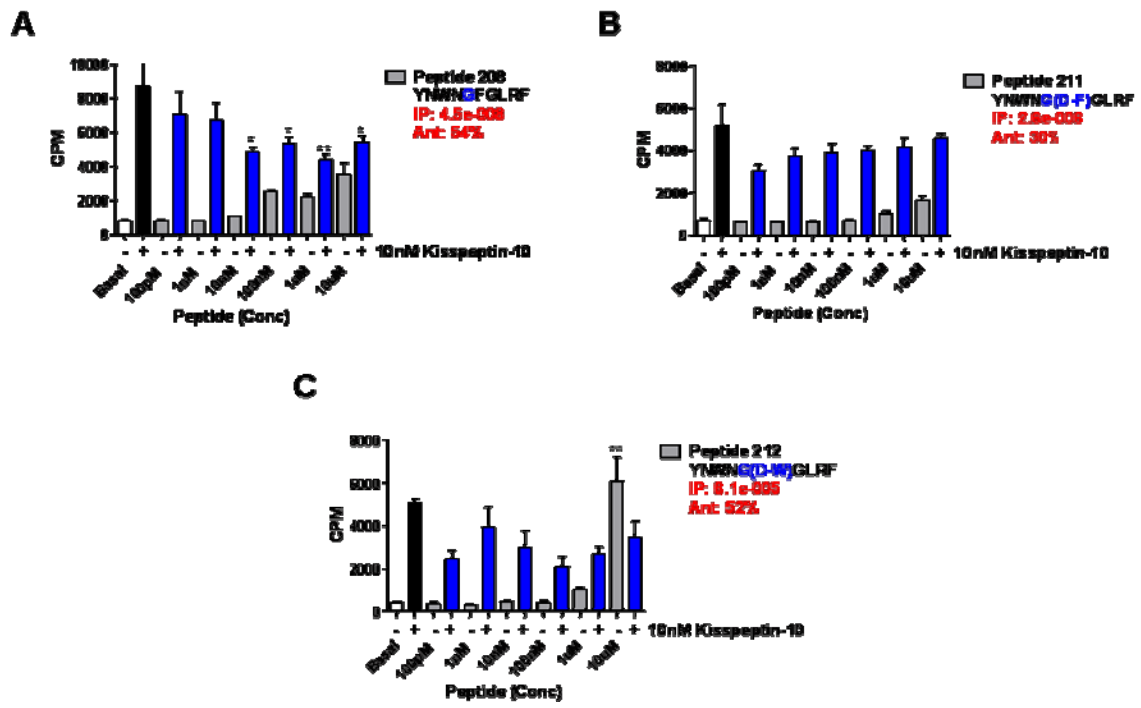

Figure 39. Gly^5 causes antagonism and this is not enhanced by position 6 substitutions. Graphs showing both intrinsic IP release (light grey) and antagonism of kp-10 stimulated IP release (dark grey). IP is the concentration at which stimulation reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) Substitution of Ser^5 to Gly^5 enhances antagonism at high doses but still activates the receptor at 45nM. (B, C) Additional substitutions at position 6 also stimulate IP release but D-Phe^6 and D-Trp^6 do not increase the antagonism over peptide 208.

As Leu^8 had been shown to create antagonism in the truncated peptides, changes at this residue were tested next in combination with Gly^5. D-Leu^8 (peptide 209) did not activate the receptor but could not antagonise any further than Gly^5 alone, whereas D-Trp^8 (peptides 210 and 228) again elicited no intrinsic IP release but caused further antagonism to 64% with an apparent IC_{50} of $<1x10^{-10}$M. The acetylated version of this peptide could also inhibit kp-10 stimulation of IP by 69%. Combination of changes at positions 6 and 8 with Gly^5 (peptide 213) appeared to disrupt the structural conformation of the peptide as it could no longer bind, activate or antagonise the receptor (Fig. 40 and Table 7). This suggests that Leu^8 is important for receptor activation and that appropriate substitutions in the C-terminal region promote antagonism. Therefore, D-Trp^8 was

incorporated into further analogues along with Gly^5. To test if Gly was the best residue for antagonism at position 5, changes were made in combination with D-Trp^8. D-Trp^5 (peptide 229) slightly decreased the antagonism, however, D-Ala^5 (peptide 233) increased the antagonism to 71% but the IC_{50} was reduced to $7x10^{-7}$M. Therefore, Gly^5 is the best substitution for this position (Fig. 41 and Table 7).

Kisspeptin Analogues (CHO cells)													Dose Response		Antagonistic IP Inhibition		Possible
No.	Peptide Sequence												Bind(IC50)	IP(EC50)	IC50	% inh max	Antagonists
169		Y	N	W	N	S	F	G	L	R	F	NH_2	7.77E-09	3.90E-09	n/a	n/a	n/a
208		Y	N	W	N	G	F	G	L	R	F	NH_2	1.17E-07	4.56E-08	1.00E-07	54%	poor
209		Y	N	W	N	G	F	G	(D)-L	R	F	NH_2	2.80E-06	No IP	5.00E-08	56%	poor
210		Y	N	W	N	G	F	G	(D)-W	R	F	NH_2	5.96E-09	No IP	3.00E-09	64%	poor
211		Y	N	W	N	G	(D)-F	G	L	R	F	NH2	4.32E-06	2.57E-06	4.00E-08	30%	poor
212		Y	N	W	N	G	(D)-W	G	L	R	F	NH_2	6.30E-06	6.09E-05	2.00E-08	52%	poor
213		Y	N	W	N	G	(D)-L	G	(D)-L	R	F	NH_2	1.30E-06	No IP	n/a	0%	none
228	ac	Y	N	W	N	G	F	G	(D)-W	R	F	NH_2	1.03E-11	No IP	4.00E-08	69%	poor
229	ac	Y	N	W	N	(D)-W	F	G	(D)-W	R	F	NH_2	No Displacement	No IP	5.00E-07	50%	poor
230	**ac**	**(D)-Y**	**N**	**W**	**N**	**G**	**F**	**G**	**(D)-W**	**R**	**F**	**NH_2**	**7.29E-09**	**No IP**	**3.00E-08**	**81%**	**good**
231	ac	Y	N	(D)-W	N	G	F	G	(D)-W	R	F	NH_2	No Displacement	No IP	2.00E-08	60%	poor
232	ac	Y	(D)-N	W	N	G	F	G	(D)-W	R	F	NH_2	No Displacement	No IP	3.00E-08	67%	poor
233	ac	Y	N	W	N	(D)-A	F	G	(D)-W	R	F	NH_2	7.90E-09	No IP	7.00E-07	71%	good
234	**ac**	**(D)-A**	**N**	**W**	**N**	**G**	**F**	**G**	**(D)-W**	**R**	**F**	**NH_2**	**2.79E-09**	**No IP**	**7.00E-08**	**93%**	**good**
235	ac	Y	N	(D)-A	N	G	F	G	(D)-W	R	F	NH_2	No Displacement	No IP	1.00E-07	73%	good
236	ac	Y	(D)-A	W	N	G	F	G	(D)-W	R	F	NH_2	No Displacement	No IP	2.00E-08	71%	good
237	ac	(D)-Y	N	W	N	G	F	G	W	R	F	NH_2	n/a	6.40E-06	None	0%	none
238	ac	(D)-Y	N	W	N	S	F	G	(D)-W	R	F	NH_2	n/a	2.80E-06	None	0%	none
239	ac	(D)-Y	(D)-N	W	N	S	F	G	W	R	F	NH_2	n/a	1.60E-08	None	0%	none
240	ac	(D)-Y	(D)-N	W	N	G	F	G	W	R	F	NH_2	n/a	No IP	None	47%	poor
241	ac	(D)-Y	(D)-N	W	N	S	F	G	(D)-W	R	F	NH_2	n/a	No IP	None	32%	poor
242	ac	(D)-Y	(D)-N	W	N	G	F	G	(D)-W	R	F	NH_2	n/a	No IP	None	44%	poor
243	ac	(D)-W	N	W	N	G	F	G	(D)-W	R	F	NH2	n/a	No IP	1.00E-10	51%	poor
244	ac	(D)-F	N	W	N	G	F	G	(D)-W	R	F	NH2	n/a	No IP	5.00E-07	59%	poor
245	ac	(D)-Y	N	W	N	G	(D)-W	G	(D)-W	R	F	NH2	n/a	No IP	3.00E-07	67%	poor
246	ac	(D)-A	N	W	N	G	(D)-W	G	(D)-W	R	F	NH2	n/a	No IP	1.00E-07	64%	poor
247	ac	(D)-A	N	W	N	S	F	G	(D)-W	R	F	NH2	n/a	No IP	5.00E-09	78%	good
248	ac	(D)-A	N	W	N	G	F	G	W	R	F	NH2	n/a	No IP	5.00E-06	65%	poor
271	P	(D)-A	N	W	N	G	F	G	(D)-W	R	F	NH_2	1.56E-08	No IP	1.00E-06	62%	poor
273	**ac**	**(D)-A**	**N**	**W**	**N**	**(D)-S**	**F**	**G**	**(D)-W**	**R**	**F**	**NH2**	**1.46E-10**	**No IP**	**1.00E-10**	**80%**	**good**
274	ac	(D)-A	N	W	N	P	F	G	(D)-W	R	F	NH2	n/a	5.30E-04	n/a	24%	poor
275	ac	(D)-A	N	W	N	(D)-P	F	G	(D)-W	R	F	NH2	n/a	No IP	n/a	45%	poor
276	**ac**	**(D)-A**	**N**	**W**	**N**	**G**	**F**	**G**	**(D)-L**	**R**	**F**	**NH2**	**n/a**	**No IP**	**1.00E-12**	**85%**	**good**
277	ac	(D)-A	N	W	N	(D)-A	F	G	L	R	F	NH2	n/a	1.50E-06	1.00E-07	24%	poor
278	ac	(D)-A	N	W	N	G	F	G	L	(D)-R	F	NH2	n/a	No IP	1.00E-06	50%	poor

Table 7. Full length kisspeptin-10 analogues. Table showing apparent IC_{50} for binding and antagonism, and EC50 values for intrinsic IP release. The percentage of maximal inhibition at micromolar concentration are given along with an indication for possible antagonists. Yellow indicates a potent antagonist and blue indicates an analogue with antagonistic potential.

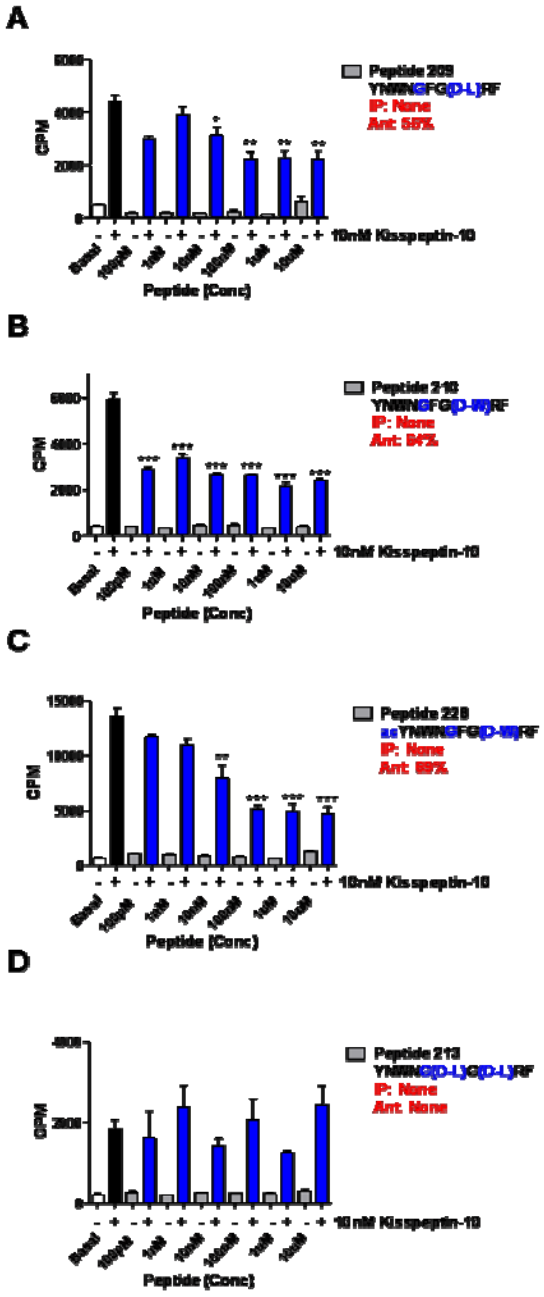

Figure 40. Gly^5 combined with Leu^8 substitution increases antagonistic effects. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) D-Leu^8 does not enhance antagonism over Gly^5 alone. (B) D-Trp^8 increases antagonism to 64% with an IC_{50} of less than 100pM. (C) Acetylation of peptide 210 further increases the antagonism to 69%. (D) Combining substitutions at Ser^5, Phe^6 and Leu^8 completely ablates the antagonistic effect seen with Gly^5 and D-Trp^8.

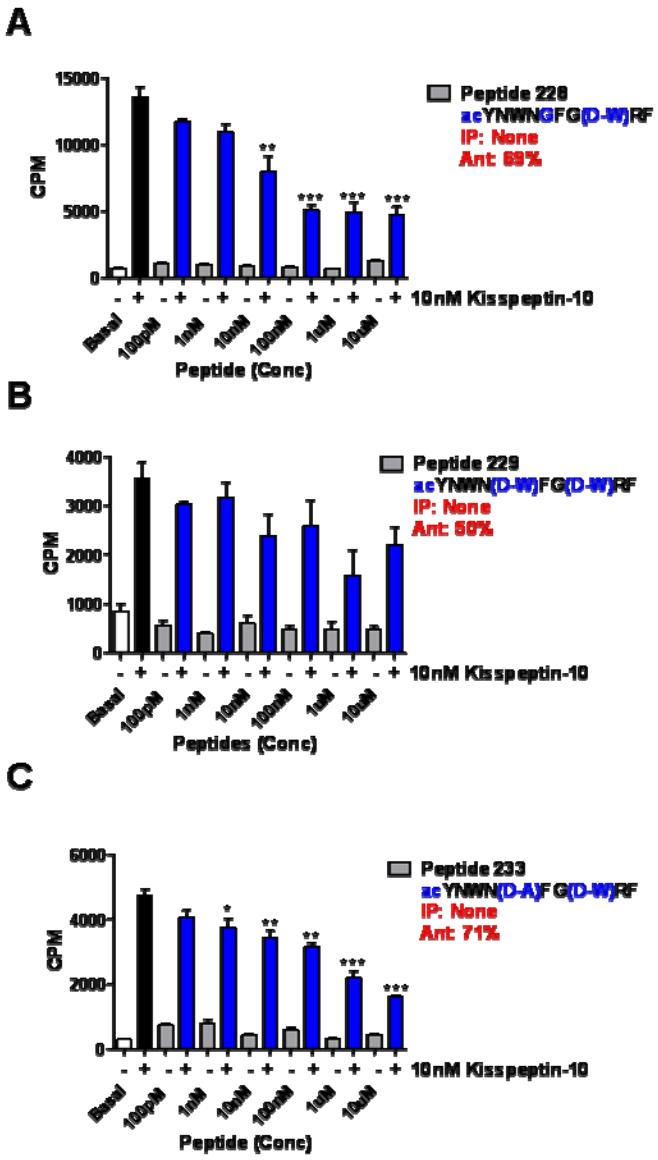

Figure 41. Small amino acid side chains and flexibility are critical for antagonists containing substitutions at Ser^5. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) Gly^5 promotes flexibility of peptides and in conjunction with D-Trp^8 antagonises by 69%. (B) Substitution of Gly^5 with a bulky D-Trp^5 reduces this antagonism to 50%. (C) However, substitution with the small amino acid, D-Ala^5 retains the antagonistic properties of peptide 228.

It was now clear that Gly^5 in combination with D-Trp^8 could antagonise receptor activation and that Leu^8 was involved in receptor activation. Therefore, to try to enhance this antagonism further, substitutions within the N-terminal region were tested in combination with these C-terminal region modifications. Changes were made at positions 1, 2 and 3. All of these analogues (peptides 230-236 and 243-4) failed to stimulate IP release and do not have intrinsic agonist activity. Position 1 substitution to D-Tyr^1 (peptide 230) significantly improved antagonism to 81%; however D-Ala^1 (peptide 234) significantly increased antagonism further to 93% with an IC_{50} of $7x10^{-8}$M. However, D-Trp^1 (peptide 243) or D-Phe^1 (peptide 244) at this position could not increase the antagonism (Fig. 42 and Table 7). This suggests that removing the charge and hydrogen bonding from this position is important for antagonism and that steric hindrance at this position is detrimental to antagonistic events. As for amino acid changes at position 2; D-Asn^2 (peptide 232) and D-Ala^2 (peptide 236) could not increase the antagonistic effects over peptide 228, therefore substitution of this residue does not appear important for antagonism of the receptor. Finally substitutions of position 3 to D-Trp^3 (peptide 231) or D-Ala^3 (peptide 235) also had no further antagonistic effect on the receptor (Fig. 43 and Table 7). Therefore, Tyr^1 is the only residue within the N-terminal region that appeared important for receptor activation and antagonism.

Next, to test whether antagonism was due to a combination of all three substituted residues or whether one was more critical, we took the two position 1 analogues and removed or changed the Gly^5 or D-Trp^8 substitution to examine the effect on the antagonistic properties of the peptide. For analogues with D-Tyr^1, modification of either of these residues completely ablated any antagonistic effect (peptides 237-8), indicating that the D-Tyr^1 has relatively little effect on the receptor antagonism alone. However, for D-Ala^1 analogues, conversion of Gly^5 back to Ser^5 (peptide 247) slightly reduced the antagonism to 78% and changing of D-Trp^8 to L-Trp^8 (peptide 248) reduced antagonism further to 65%. However, D-Leu^8 at this position (peptide 276) increased antagonism to 85%, suggesting a D-amino acid is needed at this position for antagonism; however peptide 276 does have intrinsic agonist activity (Fig. 44 and Table 7). The results

indicate that D-Ala[1] and D-Trp[8] are the most important residues for antagonism. However, Gly[5] must also play a role by keeping the peptide flexible since other substitutions at this position within peptide 234 has drastic effects except when D-Ser[5] (peptide 273) is substituted here, keeping antagonism high at 80% with an IC_{50} of $1x10^{-10}$M. Replacement with Pro[5] (peptide 274), D-Pro[5] (peptide 275) or D-Ala[5] (peptide 277) reduces antagonism to 24%, 45% and 24%, respectively (Fig. 45 and Table 7). Therefore, flexibility, possibly to allow receptor engagement appears to be the key factor in this position. Now that substitution of position 1, 5, and 8 had been shown to produce antagonists, combinations of these with position 2 substitutions were tested (peptides 239-242). However, all of these reduced antagonism. Also, introduction of D-Trp[6] into peptides 230 and 234 to increase steric hindrance at the C-terminal region (peptides 245 and 246) also reduced antagonism to around 60% (Fig. 46 and Table 7). The above data shows that amino acid changes to D-Ala[1], Gly[5] and D-Trp[8] (peptide 234) are the most critical for antagonism. However, other residues are tolerated at these positions such as D-Trp[1] in peptide 230, D-Ser[5] in peptide 273 or D-Leu[8] in peptide 276.

Effects on levels of intracellular calcium

To further investigate the effects of these antagonists, stimulation of intracellular calcium release was measured. Peptide 234 did not stimulate calcium release alone, but did antagonise kisspeptin-stimulated calcium release by 89% with an IC_{50} of $1x10^{-10}$M, further confirming its potency. Peptide 273 could also antagonise intracellular calcium release to 63% with no intrinsic stimulation and peptide 276 completely inhibited the intracellular calcium secretion (Fig. 47/Table 8). To confirm these were specific effects, three analogues that had reduced antagonism of IP were also tested, peptides 274, 275 and 277 only antagonised by 28%, 27% and 22%, respectively, confirming the effects above are specific (Table 8). Overall, four antagonists have been created (peptide 230, 234, 273 and 276) from this research and it has identified the residues important for receptor binding (Asn[2], Trp[3], Phe[6], Arg[9] and Phe[10]) and for receptor activation (Tyr[1] and Leu[8]). As peptide 234 was the most potent antagonist created *in vitro* this was taken forward into *in vivo* testing.

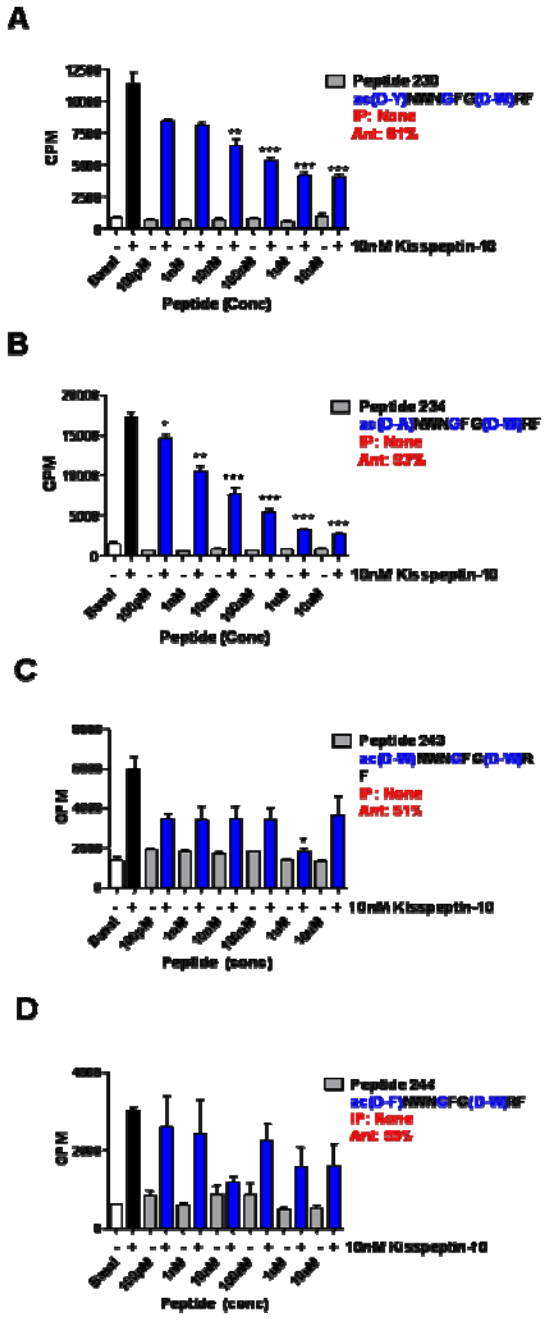

Figure 42. Amino acid substitutions at position 1 increase antagonism. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation has reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) D-Tyr at position 1 increases antagonism to 81% with no intrinsic IP release. (B) D-Ala at position 1 increases antagonism to 93% with and IC_{50} of 70nM and no intrinsic IP production. (B) D-Trp^1 and (C) D-Phe^1 does not increase antagonism showing steric hindrance in this area is not tolerated.

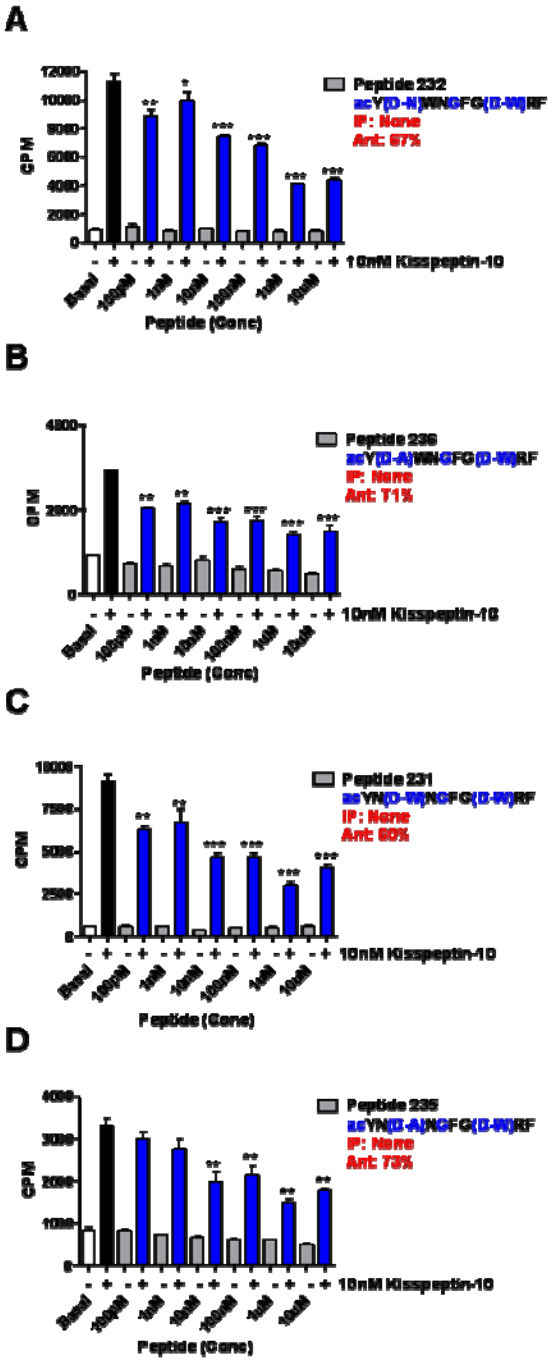

Figure 43. Positions 2 and 3 are not involved in receptor activation. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation has reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) D-Asn and (B) D-Ala at position 2 do not increase antagonism over peptide 228. (C) D-Trp and (D) D-Ala at position 3 do not increase antagonism over peptide 228.

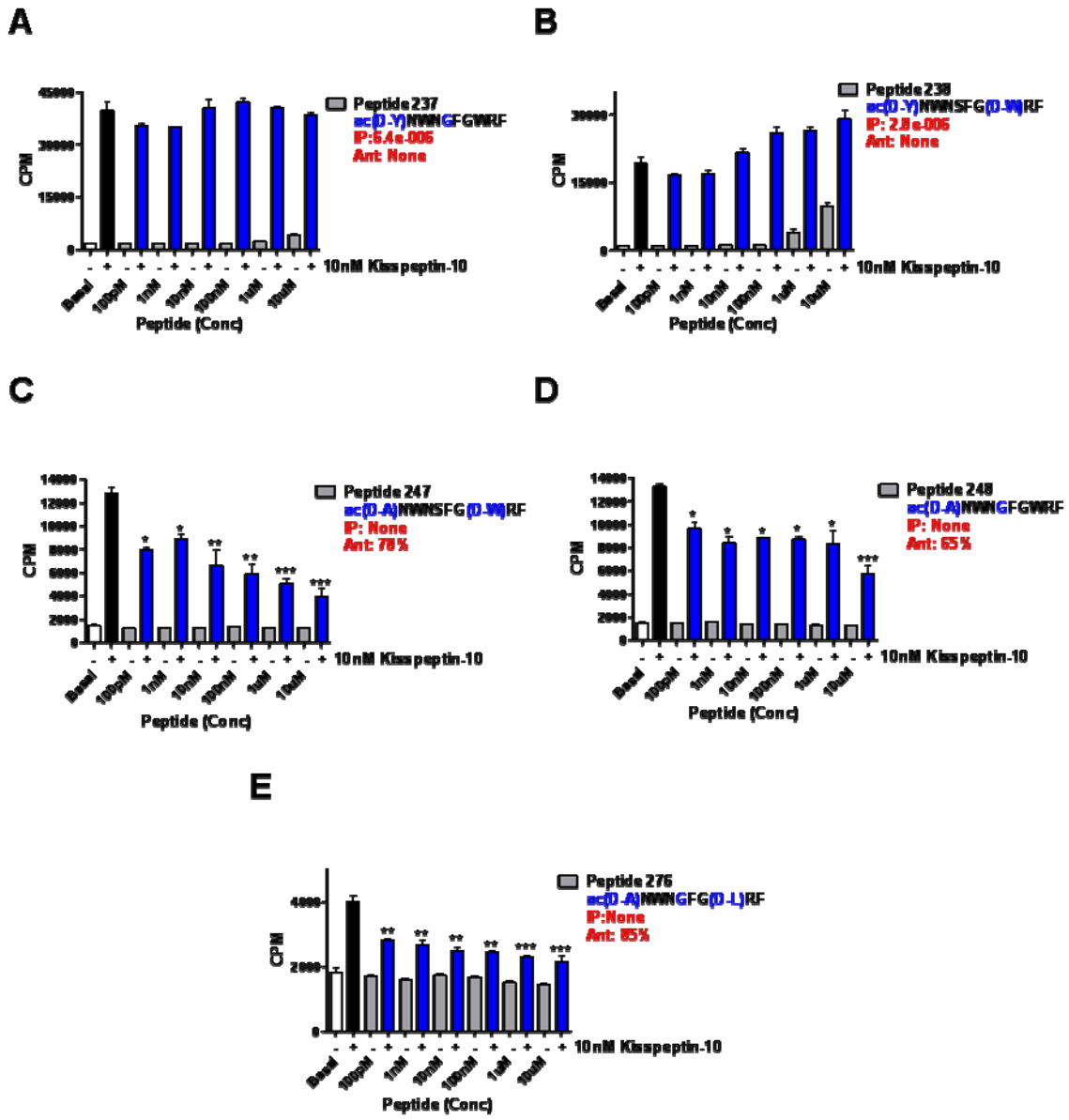

Figure 44. Removal of Gly^5 or D-Trp^8 reduces antagonism. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation has reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A, B) Removal of D-Trp^8 or Gly^5 from analogues with D-Tyr^1 completely ablates antagonism. (C, D) Removal of Gly^5 or D-Trp^8 form D-Ala^1 analogues reduce antagonism to 78% and 65%, respectively. (E) This can be increased by introducing D-Leu^8 to 85%.

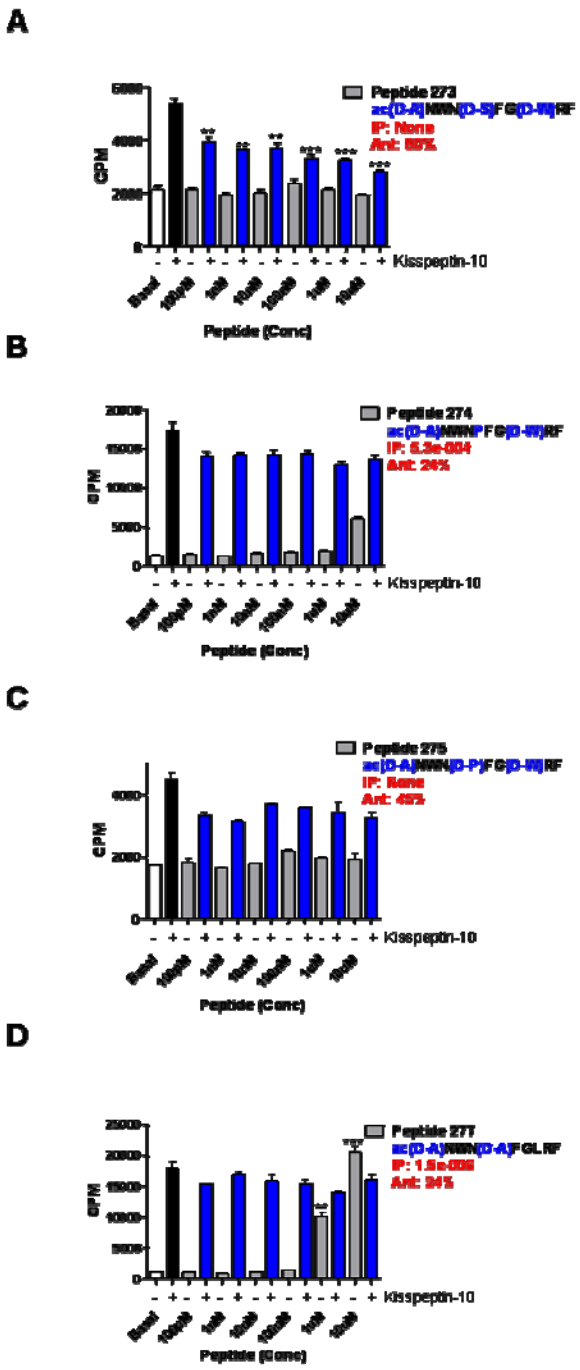

Figure 45. Glycine is the most effective residue at position 5. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation has reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. (A) D-Ser at position 5 is tolerated and antagonises by 80% with an IC_{50} of 0.1nM. (B, C) Pro or D-Pro at position 5 reduces antagonism to 24% and 45%, respectively. (D) D-Ala at position 5 reduces antagonism to 24%.

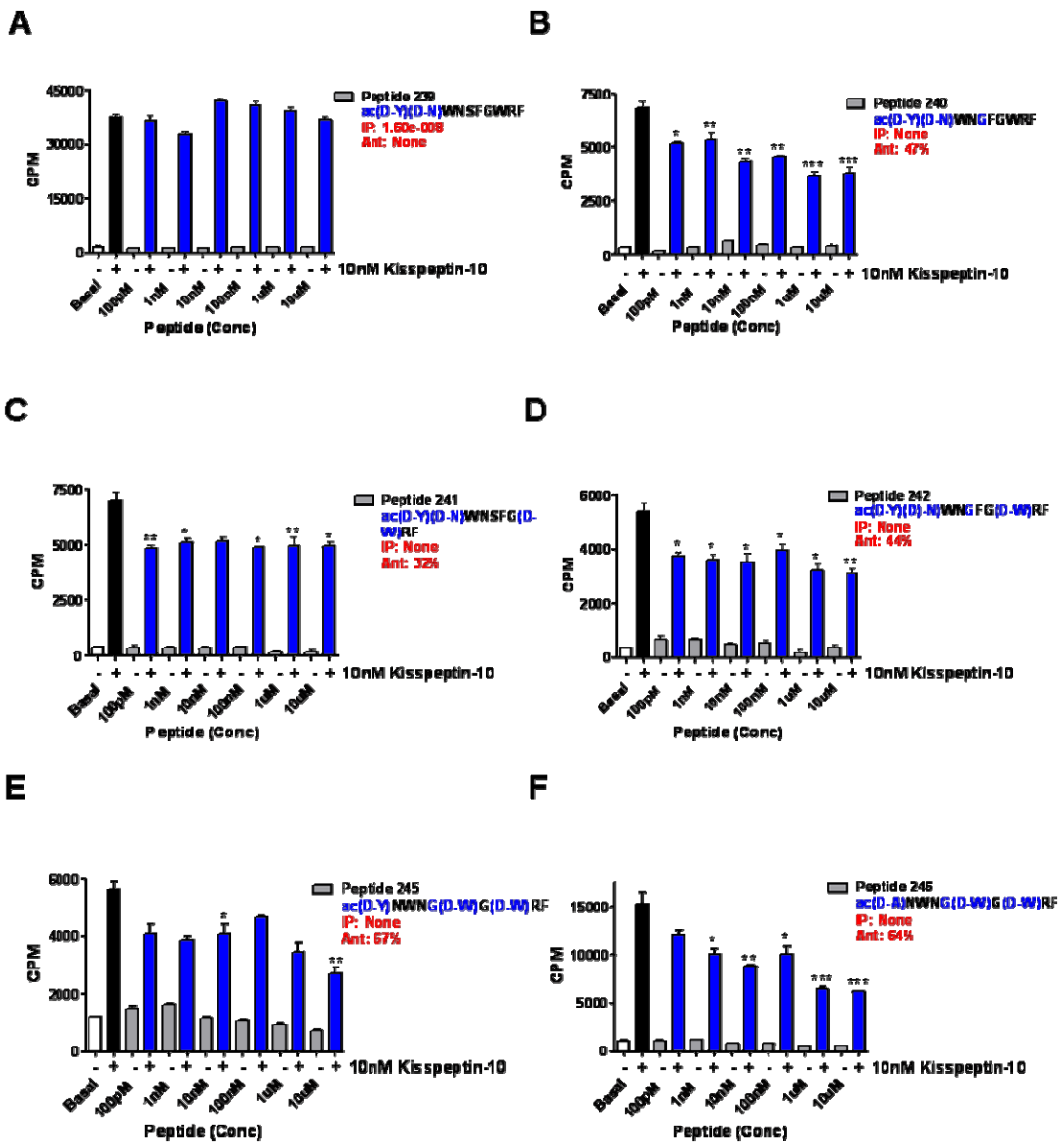

Figure 46. Double substitutions in the N-terminus and D-Trp^6 reduce antagonistic effects. Graphs showing both intrinsic IP release (grey) and antagonism of kp-10 stimulated IP release (blue). IP is the concentration at which stimulation has reached 50% of the maximal (EC_{50}) and Ant is the percentage for maximal antagonism at micromolar doses. Peptides were substituted with D-Tyr^1 and D-Asn^2 (A) without Gly^5 or D-Trp^8 completely ablating antagonism. (B) Introduction of D-Trp^8 increased antagonism to 47% and (C) introduction of Gly^5 also increased antagonism to 32%. (D) When both Gly^5 and D-Trp^8 were added back, antagonism increased to 44%. (E, F) Introduction of D-Trp^6 into peptides 230 and 234 decreased antagonism to 67% and 64%, respectively.

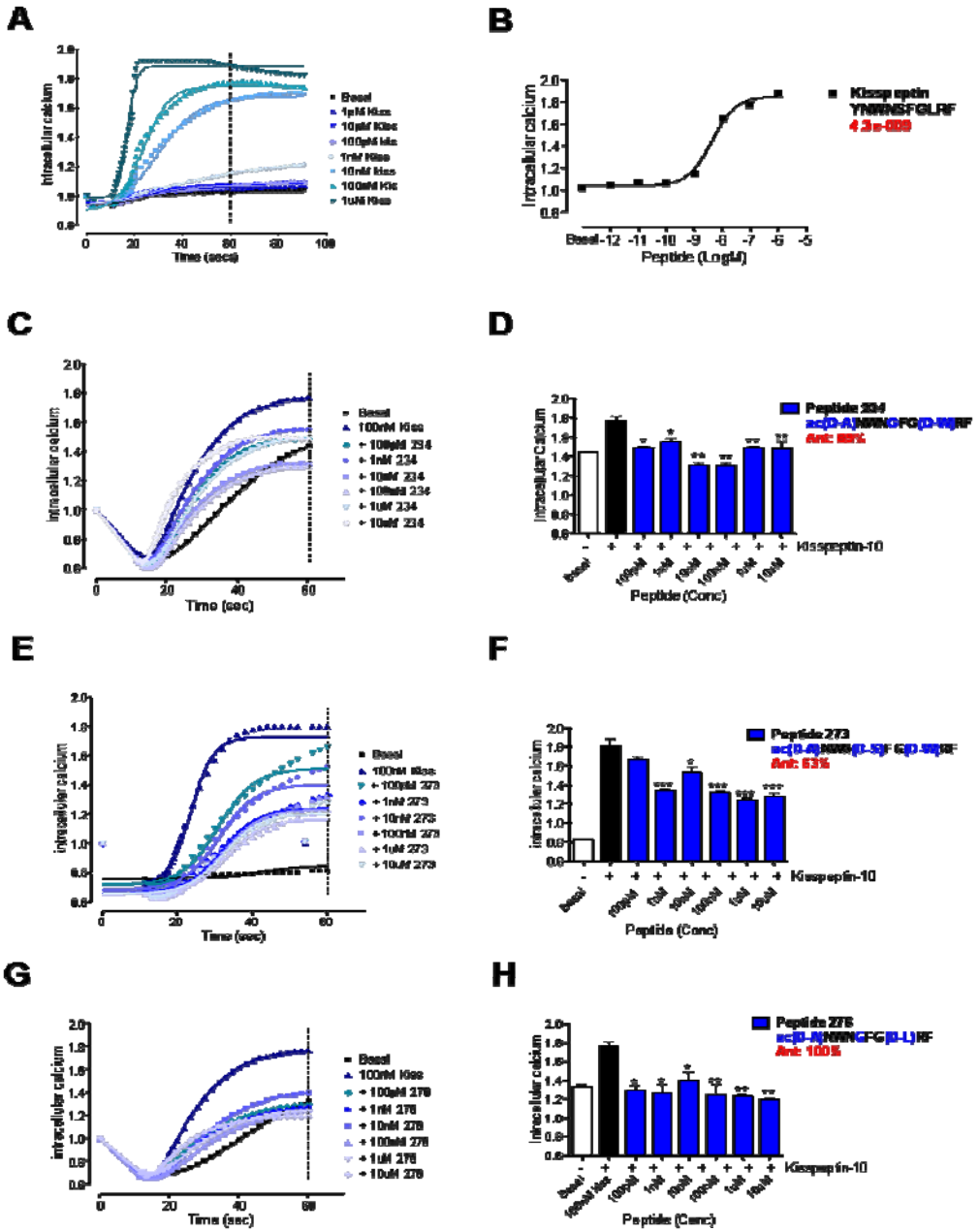

Figure 47. Effects of selected peptides on the elevation of intracellular calcium by kp-10 in CHO/gpr-54 cells. Graphs show antagonism of kp-10 stimulated calcium at 60 seconds. Ant = the maximal antagonism achieved at micromolar concentrations. (A) Raw calcium transients for kisspeptin-10 showing 60 minute time point (dotted line). (B) Quantification of kisspeptin calcium transients. (C) Peptide 234 calcium transients showing antagonism of kp-10 stimulated calcium (D) Quantification of peptide 234 transients showing 89% antagonism. (E) Peptide 273 calcium transient showing partial antagonism of kp-10 stimulated calcium by 63%. (F) Quantification of peptide 273 calcium transients. (G) Peptide 276 calcium transients showing complete antagonism of kp-10 stimulated calcium. (H) Quantification of peptide 276 calcium transients showing 100% antagonism.

No.	Kisspeptin Analogues (CHO cells) Peptide Sequence										Dose Response		Antagonistic IP Inhibition		Antagonistic Ca^{2+} Inhibition		Possible Antagonists
											Bind(Kd)	IP(EC50) Ca^{2+}(EC50)	IC50	% inh max	IC50	% inh max	
Kiss	Y	N	W	N	S	F	G	L	R	F	NH_2	7.70E-09	3.90E-09	n/a	n/a	n/a	n/a
186			ac	N	S	F	G	L	R	F	NH_2	1.85E-06	5.90E-07	n/a	agonist	n/a	n/a	poor
187	Y	N	W	N	S	F	G	L	R	W	NH_2	1.50E-07	4.00E-08	n/a	36%	n/a	n/a	poor
188				ac	F	G	L	R	F	W	NH_2	1.43E-07	8.70E-08	n/a	agonist	n/a	n/a	poor
189				ac	F	G	L	R	W	NH_2		9.46E-07	3.60E-08	n/a	agonist	n/a	n/a	poor
190				ac	F	G	(D)-F	R	W	NH_2	No displacement		2.00E-06	n/a	agonist	n/a	n/a	poor
191				ac	F	G	(D)-L	R	W	NH_2		9.39E-06	1.80E-06	n/a	agonist	n/a	n/a	poor
200				ac	F	G	L	(D)-R	W	NH_2	No displacement		No IP	n/a	0%	n/a	n/a	none
201				ac	F	G	A	R	W	NH_2		4.02E-03	No IP	7.00E-09	67%	n/a	n/a	poor
202				ac	A	G	L	R	W	NH_2	No displacement		3.40E-05	n/a	0%	n/a	n/a	none
203				ac	(D)-F	G	L	R	W	NH_2	No displacement		9.10E-06	n/a	40%	n/a	n/a	poor
206				ac	F	G	(D)-W	R	F	NH_2	No displacement		No IP	5.00E-09	71%	n/a	n/a	good
207				ac	F	G	(D)-F	R	F	NH_2	No displacement		No IP	3.00E-09	52%	n/a	n/a	poor
208	Y	N	W	N	G	F	G	L	R	F	NH_2	1.17E-07	4.56E-08	n/a	1.00E-07	54%	n/a	poor
209	Y	N	W	N	G	F	G	(D)-L	R	F	NH_2	2.80E-06	No IP	n/a	5.00E-08	56%	n/a	poor
210	Y	N	W	N	G	F	G	(D)-W	R	F	NH_2	5.97E-09	No IP	n/a	3.00E-09	64%	n/a	poor
211	Y	N	W	N	G	(D)-F	G	L	R	F	NH2	4.32E-06	2.57E-06	n/a	4.00E-08	30%	n/a	poor
212	Y	N	W	N	G	(D)-W	G	L	R	F	NH_2	6.30E-06	6.09E-05	n/a	2.00E-08	52%	n/a	poor
213	Y	N	W	N	G	(D)-L	G	(D)-L	R	F	NH_2	1.30E-06	No IP	n/a	n/a	0%	n/a	none
228	ac	Y	N	W	N	G	F	G	(D)-W	R	F	NH_2	1.03E-11	No IP	n/a	4.00E-08	69%	n/a	poor
229	ac	Y	N	W	N	(D)-W	F	G	(D)-W	R	F	NH_2	No displacement		No IP	n/a	5.00E-07	50%	n/a	poor
230	**ac**	**(D)-Y**	**N**	**W**	**N**	**G**	**F**	**G**	**(D)-W**	**R**	**F**	**NH_2**	**7.29E-09**	**No IP**	**n/a**	**3.00E-08**	**81%**	**n/a**	**n/a**	**good**
231	ac	Y	N	(D)-W	N	G	F	G	(D)-W	R	F	NH_2	No displacement		No IP	n/a	2.00E-08	60%	n/a	n/a	poor
232	ac	Y	(D)-N	W	N	G	F	G	(D)-W	R	F	NH_2	No displacement		No IP	n/a	3.00E-08	67%	n/a	n/a	poor
233	ac	Y	N	W	N	(D)-A	F	G	(D)-W	R	F	NH_2	7.90E-09		No IP	None	7.00E-07	71%	5.00E-12	83%	good
234	**ac**	**(D)-A**	**N**	**W**	**N**	**G**	**F**	**G**	**(D)-W**	**R**	**F**	**NH_2**	**2.79E-09**	**No IP**	**None**	**7.00E-08**	**93%**	**1.00E-11**	**89%**	**good**
235	ac	Y	N	(D)-A	N	G	F	G	(D)-W	R	F	NH_2	No displacement		No IP	n/a	1.00E-07	73%	n/a	n/a	good
236	ac	Y	(D)-A	W	N	G	F	G	(D)-W	R	F	NH_2	No displacement		No IP	n/a	2.00E-08	71%	n/a	n/a	good
237	ac	(D)-Y	N	W	N	G	F	G	W	R	F	NH_2	n/a	6.40E-06	n/a	None	0%	n/a	n/a	n/a	none
238	ac	(D)-Y	N	W	N	S	F	G	(D)-W	R	F	NH_2	n/a	2.80E-06	n/a	None	0%	n/a	n/a	n/a	none
239	ac	(D)-Y	(D)-N	W	N	S	F	G	W	R	F	NH_2	n/a	1.60E-08	n/a	None	0%	n/a	n/a	n/a	none
240	ac	(D)-Y	(D)-N	W	N	G	F	G	W	R	F	NH_2	n/a	No IP	n/a	None	47%	n/a	n/a	n/a	poor
241	ac	(D)-Y	(D)-N	W	N	S	F	G	(D)-W	R	F	NH_2	n/a	No IP	n/a	None	32%	n/a	n/a	n/a	poor
242	ac	(D)-Y	(D)-N	W	N	G	F	G	(D)-W	R	F	NH_2	n/a	No IP	n/a	None	44%	n/a	n/a	n/a	poor
243	ac	(D)-W	N	W	N	G	F	G	(D)-W	R	F	NH2	n/a	No IP	n/a	1.00E-10	51%	n/a	n/a	n/a	poor
244	ac	(D)-F	N	W	N	G	F	G	(D)-W	R	F	NH2	n/a	No IP	n/a	5.00E-07	59%	n/a	n/a	n/a	poor
245	ac	(D)-Y	N	W	N	G	(D)-W	G	(D)-W	R	F	NH2	n/a	No IP	n/a	3.00E-07	67%	n/a	n/a	n/a	poor
246	ac	(D)-A	N	W	N	G	(D)-W	G	(D)-W	R	F	NH2	n/a	No IP	n/a	1.00E-07	64%	n/a	n/a	n/a	poor
247	ac	(D)-A	N	W	N	S	F	G	(D)-W	R	F	NH2	n/a	No IP	n/a	5.00E-09	78%	n/a	n/a	n/a	good
248	ac	(D)-A	N	W	N	G	F	G	W	R	F	NH2	n/a	No IP	n/a	5.00E-06	65%	n/a	n/a	n/a	poor
271	P	(D)-A	N	W	N	G	F	G	(D)-W	R	F	NH_2	1.56E-08	No IP	n/a	1.00E-06	62%	1.00E-11	100%	n/a	poor
273	**ac**	**(D)-A**	**N**	**W**	**N**	**(D)-S**	**F**	**G**	**(D)-W**	**R**	**F**	**NH2**	**1.46E-10**	**No IP**	**None**	**1.00E-10**	**80%**	**1.00E-08**	**63%**	**good**
274	ac	(D)-A	N	W	N	P	F	G	(D)-W	R	F	NH2	n/a	5.30E-04	7.30E-09	n/a	24%	n/a	28%	n/a	poor
275	ac	(D)-A	N	W	N	(D)-P	F	G	(D)-W	R	F	NH2	n/a	No IP	None	n/a	45%	n/a	27%	n/a	poor
276	**ac**	**(D)-A**	**N**	**W**	**N**	**G**	**F**	**G**	**(D)-L**	**R**	**F**	**NH2**	**n/a**	**No IP**	**None**	**1.00E-12**	**85%**	**1.00E-11**	**100%**	**n/a**	**good**
277	ac	(D)-A	N	W	N	(D)-A	F	G	L	R	F	NH2	n/a	1.50E-06	9.40E-08	1.00E-07	24%	n/a	22%	n/a	poor
278	ac	(D)-A	N	W	N	G	F	G	L	(D)-R	F	NH2	n/a	No IP	None	1.00E-06	50%	n/a	21%	n/a	poor

Table 8. Table detailing all kisspeptin-10 peptide analogues used in this study. Table showing the peptide analogues tested with results for binding and stimulation of IP and Calcium release. Antagonistic results for IP and Calcium are also shown. The four antagonists found are highlighted in yellow and other promising analogues in blue.

3.2.4. Kisspeptin antagonist inhibits GnRH pulses in rodents and monkeys

Selected antagonists characterised *in vitro*, where then tested for *in vivo* activity by collaborators with specialised models. For these studies the antagonist with the highest potency and efficiency *in vitro* was utilised, this is peptide 234. Firstly, the effect of peptide 234 on GnRH neuron firing was investigated in mouse brain slices. These studies were performed by Sue Moenter and Justyna Pielecka-Fortuna in the University of Virginia, USA. Then the effects on GnRH pulsatility in the female rhesus monkey were investigated by Ei Terasawa and Kathryn Guerriero in the University of Wisconsin-Madison, USA.

As has been demonstrated previously (Han et al., 2005; Pielecka-Fortuna et al., 2008), 1nM kisspeptin-10 markedly increased GnRH neuron firing activity in mice with GFP-labelled GnRH neurons (Fig. 48). Under these experimental conditions, there was no effect on GnRH neuron firing activity of peptide 234 alone (1nM pre 0.2 ± 0.1 Hz, post 0.3 ± 0.1Hz, n=5, $P>0.05$; 10nM pre 0.3 ± 0.2 Hz, post 0.3 ± 0.2Hz, n=6, $P>0.05$; 100nM pre 0.5 ± 0.1Hz, 0.6 ± 0.1 Hz, n=7, $P>0.05$, paired t test). In contrast to the lack of effect of peptide 234 on basal firing, pre-treatment with peptide 234 blocked the response to 1nM kisspeptin-10 ($P<0.001$ for all doses) compared to cells treated with kisspeptin-10 alone (Fig. 48).

Since peptide 234 inhibited GnRH neuronal firing we investigated with collaborators whether it also inhibited GnRH release in pubertal female rhesus monkeys using methods previously described (Frost et al., 2008; Keen et al., 2008). Infusion of 10nM peptide 234 over 30 min through a microdialysis probe located in the stalk-median eminence region promptly and consistently suppressed GnRH pulses as well as mean GnRH levels but did not affect basal levels (Fig. 49). In contrast, vehicle infusion through the probe did not cause any significant changes in GnRH release (Fig. 49). The peptide 234-induced GnRH suppression was significantly different from values prior to peptide 234 infusion as well as those from the vehicle control (for both $p<0.05$). Based

on previous assessment that the dialysis membrane passes ~10% of peptides with a similar size to 234 (Frost et al., 2008), it is estimated that the concentration of peptide 234 in the stalk-median eminence region was 1nM.

These results show for the first time that endogenous kisspeptin via gpr-54 can directly influence GnRH neuron firing and can effect GnRH secretion by regulating pulse amplitude. These results confirm the findings of the previous studies using exogenous kisspeptin delivered via central and peripheral methods, which showed that kisspeptin could stimulate GnRH neuron firing and GnRH secretion. As the antagonist effects occur at doses as low as 1nM this confirms the potency of the antagonist, peptide 234 *in vivo*.

3.2.5. Kisspeptin antagonist reduces LH levels in rodents and sheep

Having shown peptide 234 inhibits GnRH neuron firing and release of GnRH, we investigated if this translated into an inhibition of LH secretion. Two rodent models were utilised to investigate LH secretion into the circulation and a sheep model was used to investigate LH pulsatility. Firstly, intact rats were used to investigate the effect of kisspeptin on LH secretion and secondly castrated rats were utilised to investigate negative feedback effects by Manuel Tena-Sempere and Rafael Pineda at the University of Cordoba, Spain. These effects on LH secretion were also investigated in intact and castrated mice by Robert Steiner, Alexander Kauffmann and Michelle Gottsch at the University of Washington, USA.

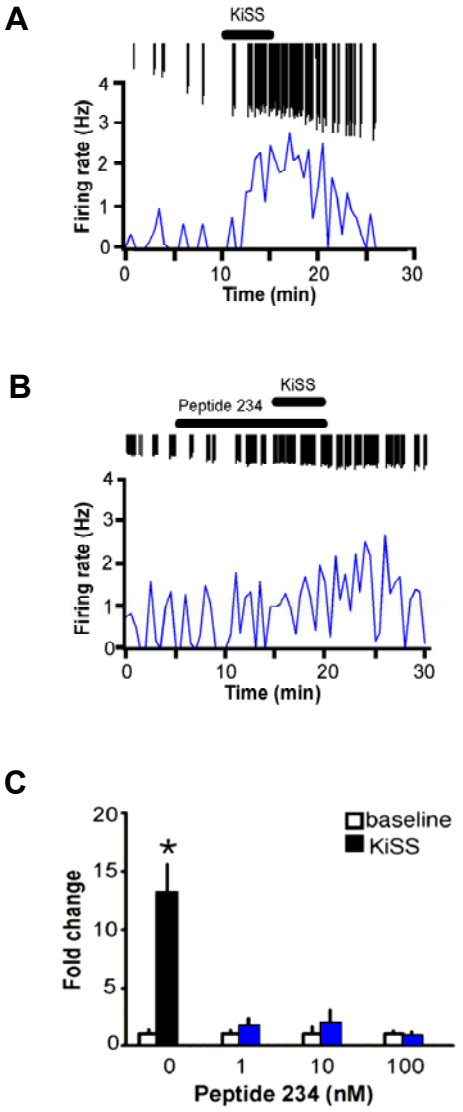

Figure 48. Peptide 234 antagonises kisspeptin-10 excitation of mouse GnRH neurons. Representative traces of GnRH neuronal firing rate over time for patch-clamped GFP-labelled GnRH neurons within slices of mouse brain. (A) Increased GnRH firing rate after 1nM kisspeptin-10 (bar). Downward spikes are individual action currents. (B) Inhibition of kisspeptin-10 (1nM) stimulation by peptide 234 (1nM, bar). (C) Summary bar graph showing mean ± SEM fold change in firing rate during baseline (white bars) and kisspeptin-10 (black/blue bars) kisspeptin-10 significantly increased firing activity of GnRH neurons (n=7, * p< 0.002). Response to kisspeptin-10 was significantly reduced with the presence of 1, 10, 100nM peptide 234 (1nM n=5, 10nM n=6, 100nM n=7, p<0.001 all groups).

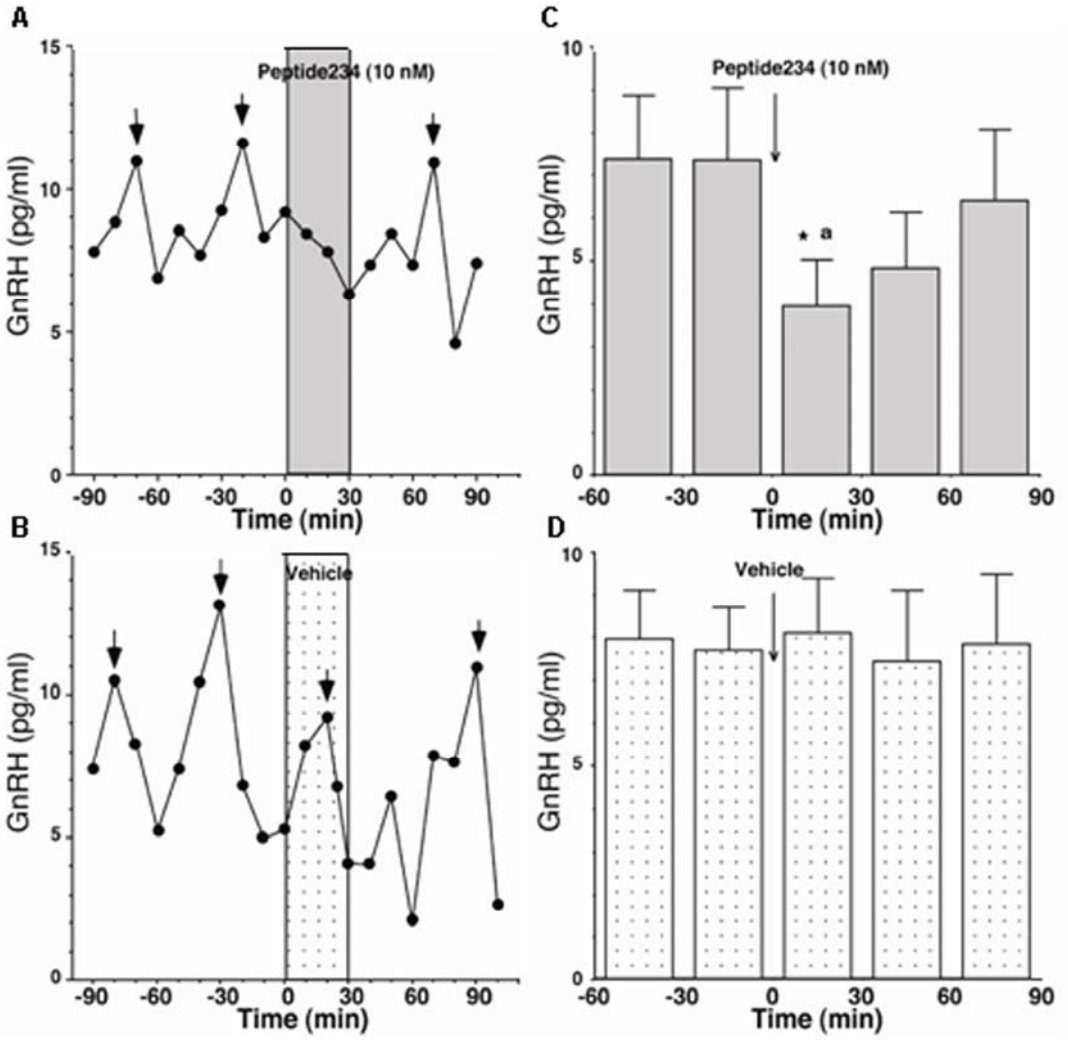

Figure 49. Peptide 234 suppresses GnRH release *in vivo* in female rhesus monkeys. Representative cases from the effects of peptide 234 on GnRH release and group mean (±SEM, n=6) are shown. (A) Pulsatile GnRH release in the hypothalamus was suppressed by 10nM peptide 234 infusion to the stalk-median eminence regions (dark shaded bar). Short arrows indicate GnRH peaks identified by the PULSAR algorithm (Merriam and Wachter, 1982). (B) In contrast, vehicle infusion as a control did not cause any significant changes in GnRH release (light shaded bar). (C) Data analysis indicated that peptide 234 significantly ($p<0.05$) suppressed GnRH release compared to levels prior to peptide 234 as well as to the vehicle control. (D) Vehicle infusion did not cause any significant changes. The estimated concentration of peptide 234 in the stalk-median eminence region was 1nM, based on previous assessment that the dialysis membrane passes ~10% of peptides with a similar size. * $p<0.05$ vs. before peptide 234; [a] $p<0.05$ vs. control at corresponding time period.

Effects on LH secretion in the intact and castrated rat

In rats, pharmacological tests involved repeated (x3) intracerebro-ventricular (icv) injections of 1nmol peptide 234 and serial blood sampling, in order to assess potential effects of antagonists on basal LH levels. The third injection was accompanied by co-administration of a submaximal dose (100pmol) of kisspeptin-10 and a 1nmol dose of peptide 234, to monitor the ability to inhibit kisspeptin-10 stimulation of LH and testosterone via activation of GnRH neurons. Administration of 1nmol peptide 234 icv did not significantly modify basal LH levels at anytime tested after injection. Central injection of 100pmol kisspeptin-10 to vehicle-treated animals (at 120 minutes) evoked the expected rise in serum LH levels with a net LH secretory mass (Area under curve; AUC) of 527 ± 43. In spite of its lack of effect on basal LH levels, co-administration of peptide 234 blunted the LH secretory responses induced by kisspeptin-10, with a significant ($P<0.01$) reduction in the net LH secretory mass (AUC) to 328 ± 56 during the 120 minute period following co-injection of peptide 234 and kisspeptin-10 (Fig. 50). As for LH, testosterone levels at 60 minutes after combined injection of peptide 234 and kisspeptin-10 (1.58 ± 0.1) were significantly ($P<0.01$) lower than in animals injected with kisspeptin-10 alone (2.17 ± 0.2).

LH levels were elevated in castrated rats over the 240 minute monitoring period due to lack of negative feedback. Administration of 1nmol peptide 234 at 0, 60 and 120 minutes tended to reduce serum LH levels in castrated males; a reduction that reached statistical significance at 240 minutes (Fig. 50).

Effects on LH secretion in the intact and castrated mouse

In mice, peptide 234 showed strong antagonistic actions on LH levels. As with the studies in male rats, peptide 234 did not significantly alter plasma LH levels in intact male mice ($p>0.30$ versus vehicle controls; data not shown). However, two infusions of peptide 234 at 15nmol inhibited LH levels in castrated males compared with the elevated LH levels of vehicle-treated castrated males ($p<0.05$; Fig. 51). The low dose (15pmol) was ineffective. The subsequent dose-response study confirmed this finding, with all

three doses of peptide 234 (1nmol, 5nmol, 15nmol) reducing LH levels of castrated males to levels observed in intact males ($p<0.01$) relative to vehicle-treated castrates (Fig. 51). Similar to two infusions of peptide 234, a single infusion of peptide 234 (5nmol or 15nmol) eliminated the post-castration rise in LH ($p<0.05$) relative to vehicle-treated castrates (Fig. 51). The inhibition of LH levels in castrated mice after treatment with peptide 234 appears to be due to specific inhibition of kisspeptin signalling (rather than non-specific effects or effects on other neuroendocrine systems) because pre-treatment of intact mice with peptide 234 completely blocked a kisspeptin-induced rise in LH ($p<0.05$; Fig. 51).

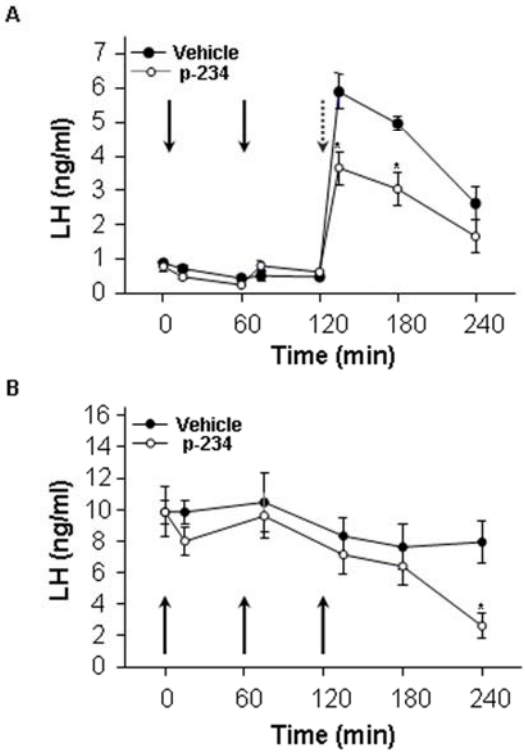

Figure 50. Peptide 234 effects on basal and kisspeptin-10-stimulated plasma LH in intact and castrated male rats. Peptide 234 (A) inhibits kisspeptin-10 induced LH secretion in intact male rats. The animals were infused icv with 1nmol peptide 234 at 0 and 60 minutes, followed by infusion of 100pmol kisspeptin-10 with 1nmol peptide 234 at 120 minutes. The peptide significantly inhibited LH production over the following 2 hours (n=10; * = $p<0.05$). (B) Castrated male rats were given three infusions of 1nmol peptide 234 at 0, 60 and 120 min, which significantly inhibited LH secretion after 240 minutes with 1nmol peptide 234 (n=10; $p<0.05$). Bars show mean ± SEM.

Effects on LH pulsatility in the sheep

Since peptide 234 was able to inhibit kp-10 stimulated and castration increased LH secretion in rat and mouse models, the next step was to discover if this was due to a decrease in pulse amplitude or frequency. This work was carried out by Iain Clarke and Jeremy Smith at Monash University, Australia. For these studies an ovariectomised sheep model was used since sheep exhibit clearly detectable LH pulses. Major secretory episodes of LH were distinguishable in control ovariectomised ewes and in treated ewes prior to peptide 234 administration (Fig. 52). LH pulse amplitude was reduced after icv administration of peptide 234 ($P<0.05$); this reduction in amplitude was so marked in some ewes that it made pulse detection difficult and precluded determining if there was an additional effect on pulse frequency. Mean LH levels were similar before and during the infusion, but reduced following peptide 234 infusion ($P<0.05$). Peptide 234 had no effect on the concentrations of prolactin or cortisol before, during or after infusion (data not shown).

The above results confirm that kisspeptin and gpr-54 via GnRH can regulate the secretion and pulsatility of LH. The differences in the time taken for peptide 234 to take effect (240 and 30 minutes) and the amount of inhibition between the rat and mouse studies may be attributable to the differences in doses used as only a low dose was utilised in the rat studies. The studies on castration where negative feedback of LH secretion has been eliminated also confirm that kisspeptin is the mediator of negative feedback since peptide 234 can completely block the increase in LH which usually occurs after castration.

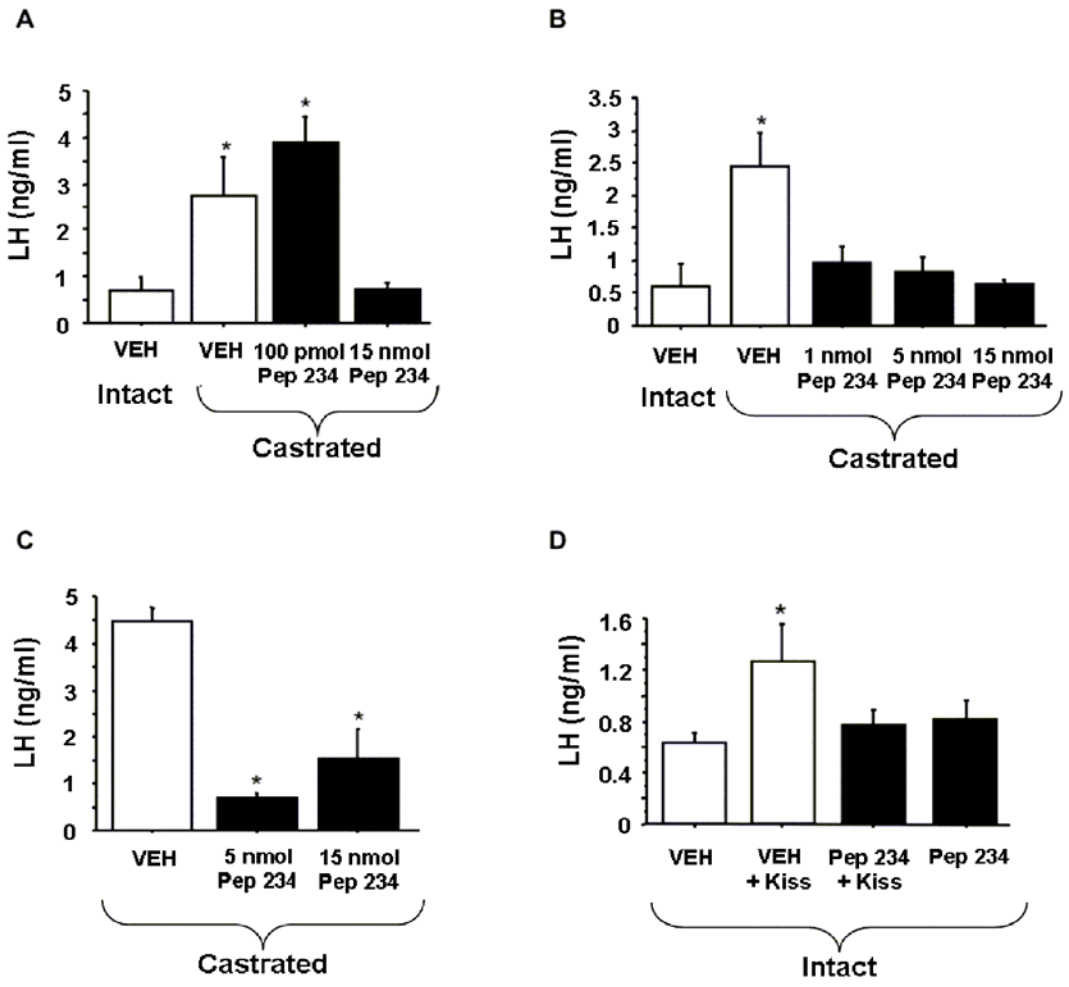

Figure 51. Peptide 234 inhibition of plasma LH in male mice. (A) Two infusions of 15nmol of peptide 234 inhibit the elevated LH levels of castrated male mice after 30 minutes. (B) Dose response graph for castrated male mice given two infusions of peptide 234 indicates that all 3 doses tested are able to inhibit the post-castration rise in LH after 30 minutes. (C) Likewise, a single infusion of 5nmol or 15nmol of peptide 234 potently inhibits LH levels of castrated mice after 30 minutes. (D) Exogenously administered kisspeptin (100fmol) is unable to stimulate LH levels in intact male mice when 100pmol peptide 234 is infused 5 minutes beforehand (n=6-8). Bars show mean ± SEM.

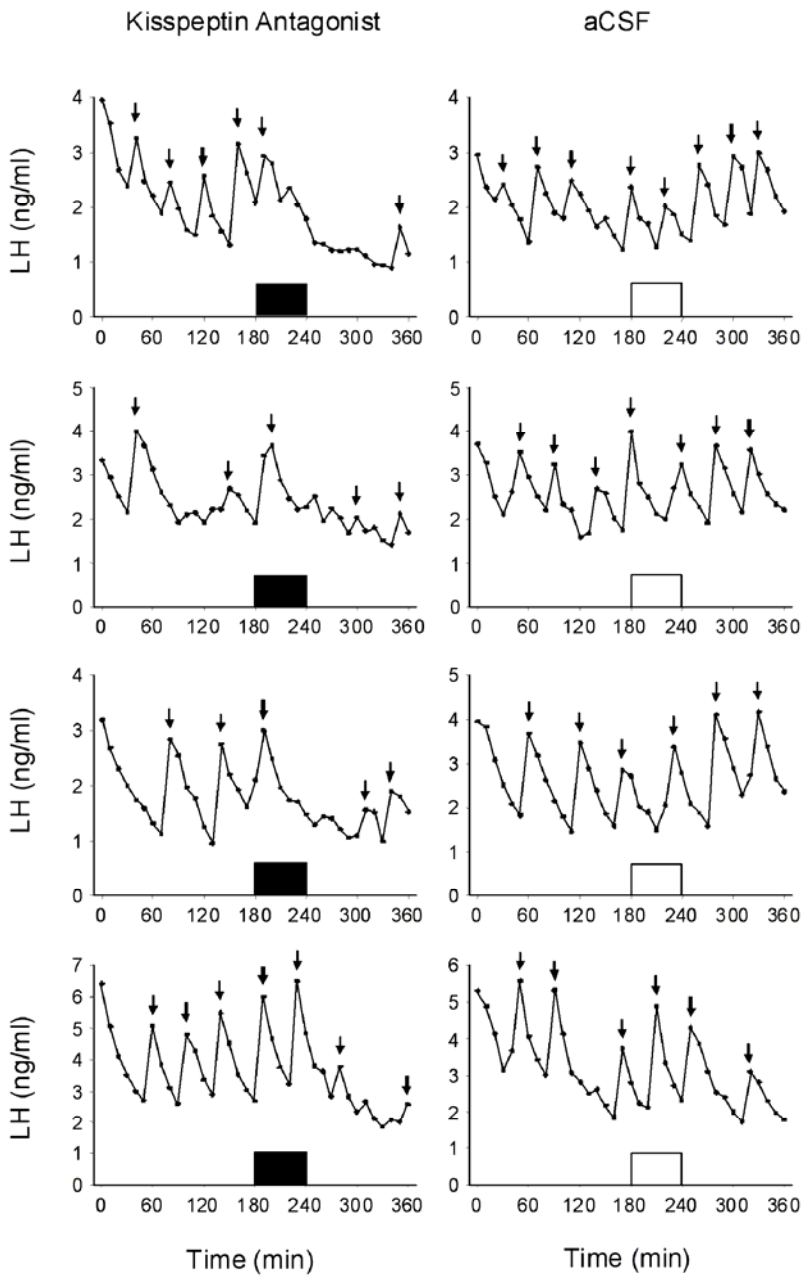

Figure 52. Central infusion of peptide 234 inhibits the secretory pulses of LH in OVX ewes. Concentrations of LH are shown in ewes treated with peptide 234 (*closed bars*) or control aCSF (artificial cerebrospinal fluid; *opened bars*). Arrows indicate LH pulses as defined in the supporting materials and methods. Analysis revealed a significant reduction in the mean LH concentration and pulse amplitude after peptide 234 infusion.

3.2.6. Kisspeptin antagonist blocks puberty and the LH surge in rodents

As peptide 234 had been shown to act as a potent antagonist at gpr-54, the effects of this antagonist on puberty onset and the pre-ovulatory surge in rats were investigated, to confirm a direct role for kisspeptin in physiological processes. This work was performed by M Tena-Sempere, R Pineda, D Garcia-Galiano, M Romero, MA Sanchez-Garrido, F Ruiz-Pino and L Pinilla at the University of Cordoba, Spain.

In order to directly assess the functional relevance of kisspeptin signalling in the central networks controlling puberty onset, a protocol of icv infusion of the antagonist, peptide 234, was applied in peri-pubertal female rats, using established methodology (Roa et al., 2008d). Thus, osmotic mini-pumps were implanted intradermally and connected to an icv cannulae into the brain to deliver a constant rate of 10nmol/24h peptide 234 during 7 days, from P30 to P36. Phenotypic and hormonal analyses were applied to monitor the progression of puberty. Infusion of peptide 234 failed to induce any noticeable changes in body weight gain throughout the period of study (Fig. 53). In contrast, icv administration of peptide 234 to peri-pubertal female rats evoked a marked delay in the timing of puberty, measured by vaginal opening. On day 36, 80% of animals infused with vehicle displayed complete canalization of vagina, whereas only 13% of the females treated with peptide 234 showed complete vaginal opening (Fig. 53). In good agreement, central infusion of peptide 234 had caused a significant reduction of ovarian and uterus weights by the end of treatment (Fig. 53). However, no significant differences in mean LH and FSH levels were detected in the circulation between controls and antagonist-treated animals on the morning of the last day (P36) of infusion (Fig. 53).

A similar protocol of central infusion of peptide 234 was applied to adult, cycling female rats in order to further define the importance of kisspeptin signalling in the generation of the pre-ovulatory surge of gonadotropins. Regularly cycling female rats, showing at least three consecutive 4-day ovarian cycles, were implanted in the morning of estrus with

osmotic mini-pumps to allow icv delivery of peptide 234, at a constant rate of 10nmol/24h. The infusion was continued until the afternoon of the following proestrus, when the animals were subjected to serial blood sampling throughout the afternoon/evening of proestrus and the morning of estrus, as described in previous experiments (Roa et al., 2008b; Roa et al., 2008c). In cyclic females infused with vehicle, 10 out of 11 animals displayed the expected pre-ovulatory surge of LH along the afternoon of proestrus, with a progressive rise of its serum concentrations between 16:00 and 20:00, followed by a drop in LH levels at the morning of estrus (Fig. 54). In striking contrast, 7 out of 9 females icv infused with peptide 234 failed to display the typical peak of LH levels at proestrus (Fig. 54). The average of the results from all animals, excluding any outliers, show a complete blockade of the pre-ovulatory LH surge in peptide 234 infused cyclic rats compared to controls (Fig. 54). LH secretion (AUC) along the afternoon/evening (14:00 to 20:00) of proestrus shows a five-fold decrease in the net secretory mass of LH during the pre-ovualtory period in animals infused with peptide 234 (Fig. 54).

FSH level (AUC) along the pre-ovulatory surge was also monitored. Animals infused with vehicle displayed a detectable increase in serum FSH levels between 16:00 and 20:00 of proestrus (primary surge), which was followed by persistently elevated FSH concentrations at the morning of estrus (secondary surge). Infusion of peptide 234 abrogated in primary FSH surge and blocked the secondary rise in FSH on the morning of estrus (Fig. 55).

These results give direct evidence for regulation of puberty onset and the LH surge by the kisspeptin system in rats. These confirm that kisspeptin and gpr-54 can regulate the timing of puberty as peptide 234 can delay vaginal opening in these animals. Peptide 234 also inhibited the LH and FSH pre-ovulatory surge in the majority of animals tested, placing kisspeptin as the main signal that regulates this process in this species. Further tests are now needed to confirm these effects in other species such as primates.

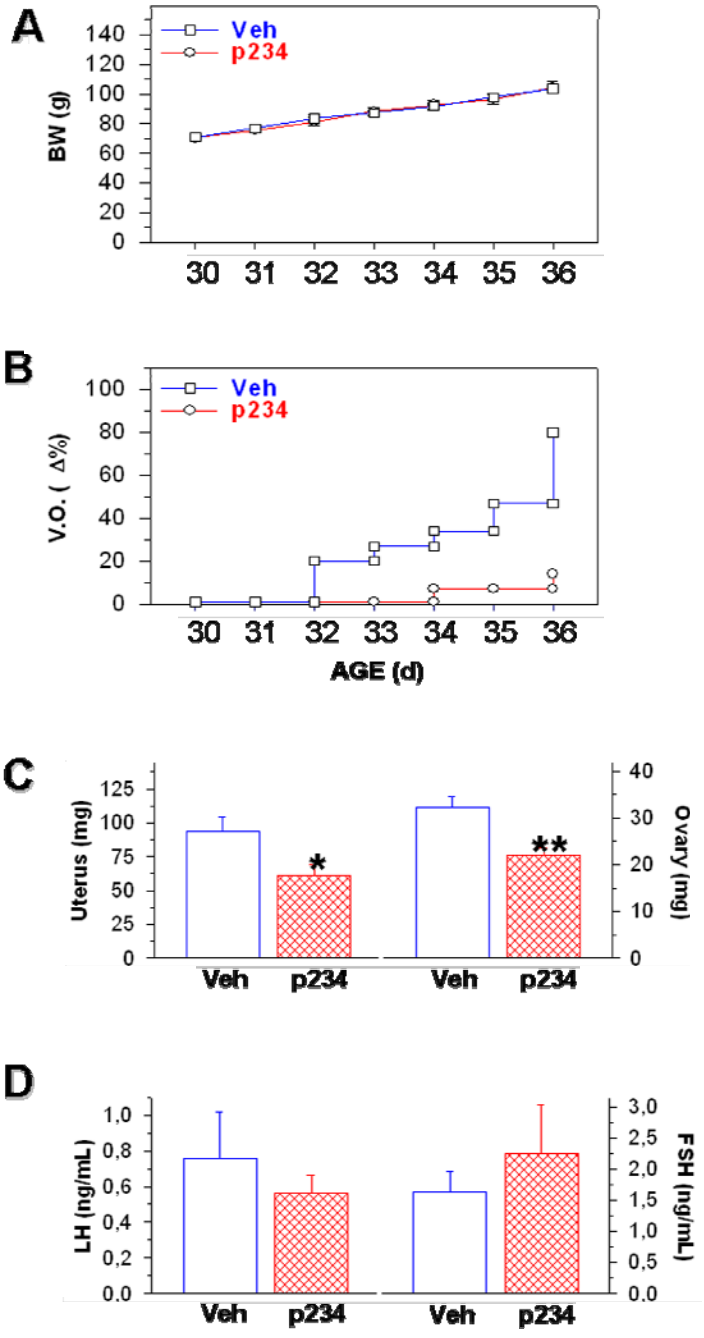

Figure 53. Effects of continuous infusion of kisspeptin antagonist on puberty onset in female rats. The impact of chronic icv. infusion of the antagonist of kisspeptin, peptide 234, to pubertal female rats (d-30 to d-36) on different indices of puberty onset is documented. Body weights (BW; **A**), vaginal opening (V.O., **B**), uterus and ovarian weights (**C**), and terminal serum LH and FSH levels (**D**) are presented for animals infused with vehicle or the antagonist. * P<0.05; ** P<0.01 vs. control (vehicle) group (Student t- test).

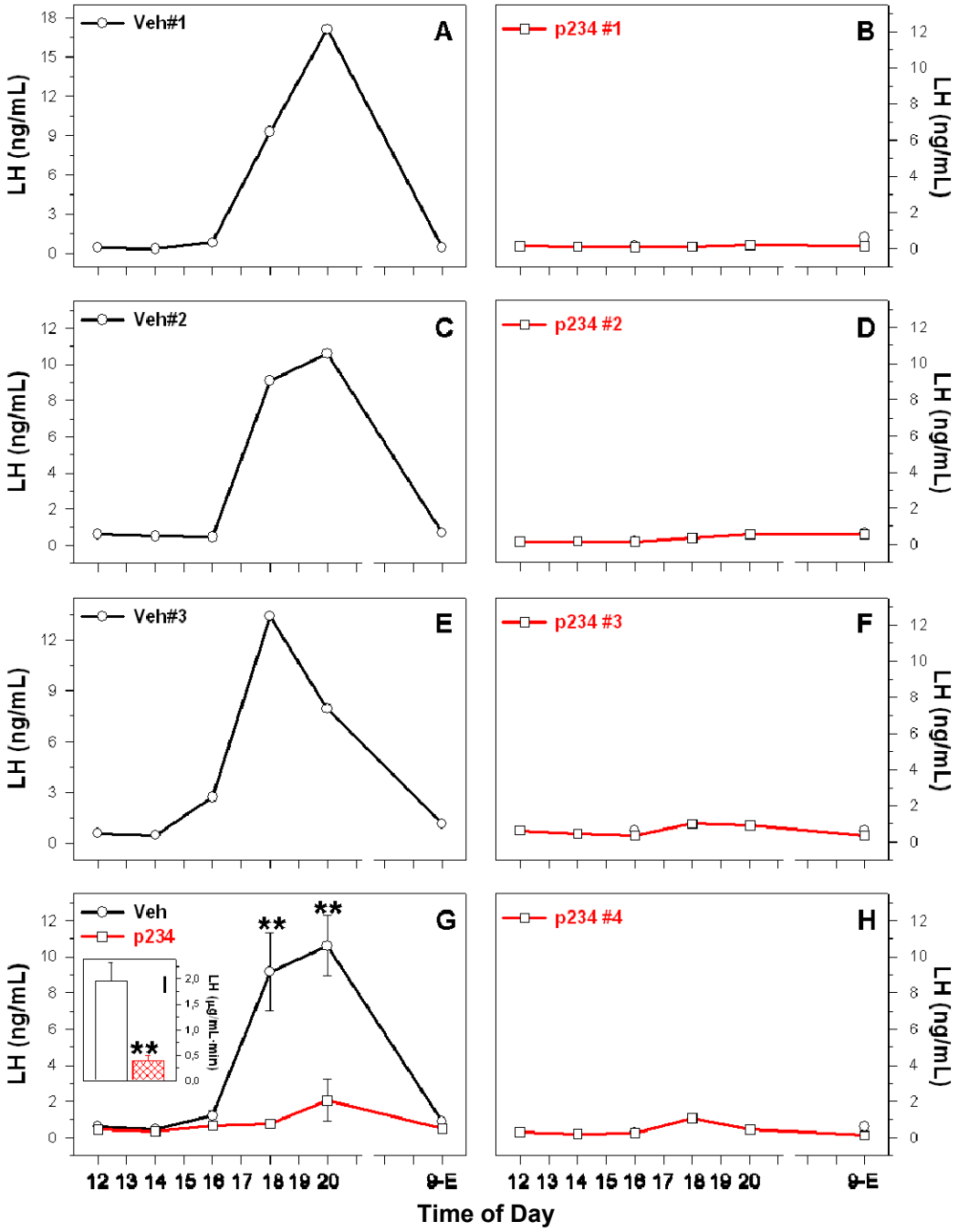

Figure 54. Effects of continuous infusion of kisspeptin antagonist on pre-ovulatory surge of LH. Individual hormonal profiles of LH secretion along the proestrus-to-estrus transition are presented from representative female rats centrally-infused with either vehicle (A,C,E) or peptide 234 (B,D,F,H). In addition, mean serum LH levels in both groups (G), as well as integrated LH secretion between 14:00 and 20:00 of proestrus (AUC; bar graph in I), are also shown. 9-E = estrus at 9am next day. For further details, see text. ** P<0.01 vs. corresponding control group.

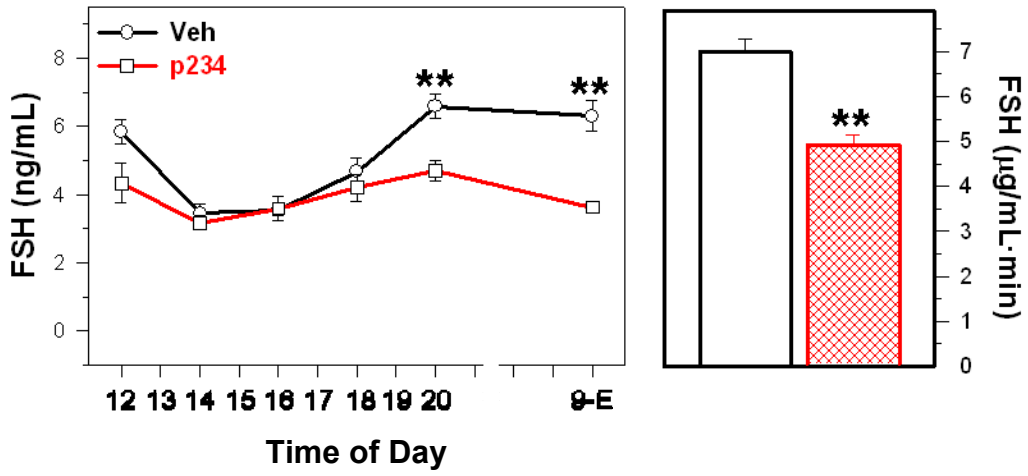

Figure 55. Effects of continuous infusion of kisspeptin antagonist on pre-ovulatory surge of FSH. Mean serum FSH levels along the proestrus-to-estrus transition female rats centrally-infused with either vehicle or peptide 234. In addition, integrated FSH secretion between 14:00 and 20:00 of proestrus (AUC) is also shown as bar graph. 9-E = estrus at 9am next day. For further details, see text. ** P<0.01 vs. corresponding control group

3.2.7. Penetratin-tagging of peptide 234

All of the above results were obtained via icv injection with peptide 234. To potentially enhance systemic activity, peptide 234 was modified by the addition of a penetratin tag in order to increase its permeability at the blood-brain barrier and this was named peptide 271. Penetratin is a 16 amino acid cationic cell-penetrating peptide with the sequence RQIKIWFQNRRMKWKK-NH2. This was derived from the homeodomain of Antennapedia protein, a drosophila transcription factor, known to be internalised and cross the cell membrane and blood-brain barrier (Derossi et al., 1998). It has since been shown that only the final seven C-terminal amino acids (RRMKWKK-NH2) are needed for this peptide to be able cross the cell membrane and blood-brain barrier (Fischer et al., 2000). For these reasons, it was these seven amino acids, RRMKWKK, that were joined to peptide 234 via a Tyr residue to create peptide 271.

Peptide 271 was first tested *in vitro* to examine if adding the tag had affected the peptides ability to bind and antagonise the receptor. Peptide 271 was found to bind to

gpr-54 with an IC_{50} of $1.56x10^{-8}$M, which is similar to peptide 234. As for activation of the receptor, peptide 271 did not induce IP production as expected for an antagonist. Finally, peptide 271 could antagonise the receptor *in vitro* by 62% (Fig. 56 and Table 6). The peptide was therefore taken into *in vivo* trials.

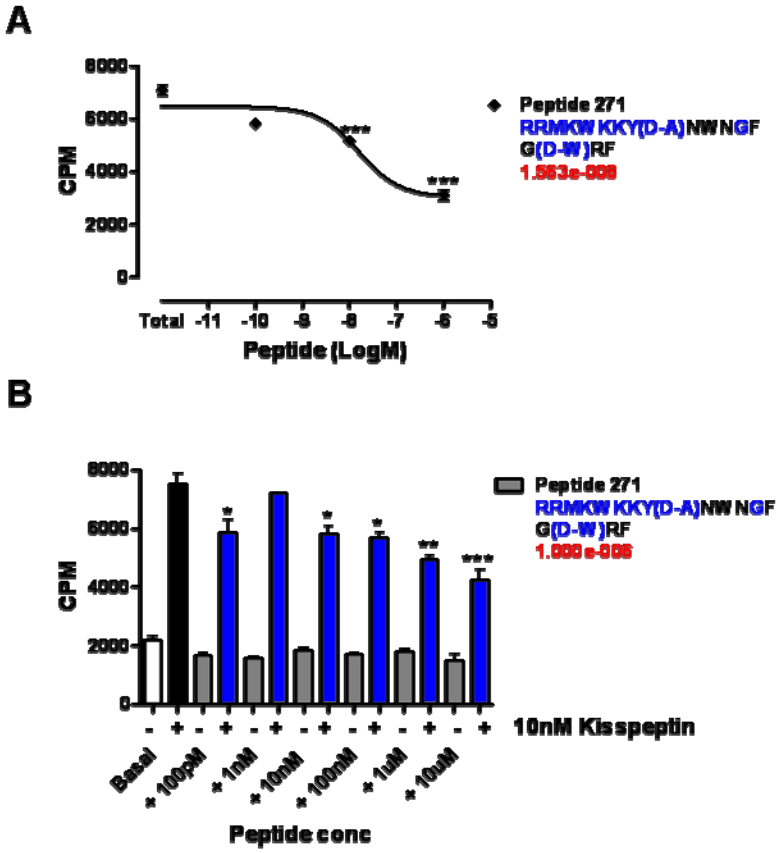

Figure 56. Penetratin tagging does not affect binding or antagonistic properties of peptide 234. (A) Peptide 271 can still bind with an IC_{50} of 15nM. (B) Peptide 271 has no intrinsic IP stimulation but can antagonise Kp-10 stimulated IP production by 62% with an IC_{50} of 1µM.

To test if peptide 271 could inhibit LH and FSH secretion when given systemically (ip) *in vivo*, a rat model was utilised by M Tena-Sempere, R Pineda, D Garcia-Galiano, M Romero, MA Sanchez-Garrido, F Ruiz-Pino and L Pinilla at the University of Cordoba, Spain. Using a similar method to that describe previously, peptide 271 (5nmol) or

vehicle were injected three times via ip injection to male rats. The third injection was accompanied by a sub-maximal injection of kisspeptin (100pmol) given icv to evoke a rise in LH or FSH. In animals given vehicle, kisspeptin was able to evoke a robust rise in LH from 120-180 minutes. FSH increased steadily over the three hour period with maximal stimulation at 240 minutes. In contrast, the animals given peptide 271 via intraperitoneal (ip) injection exhibited a reduced LH rise by around 60% and a significant decrease in the amount of FSH secretion over 2 hours (Fig. 57).

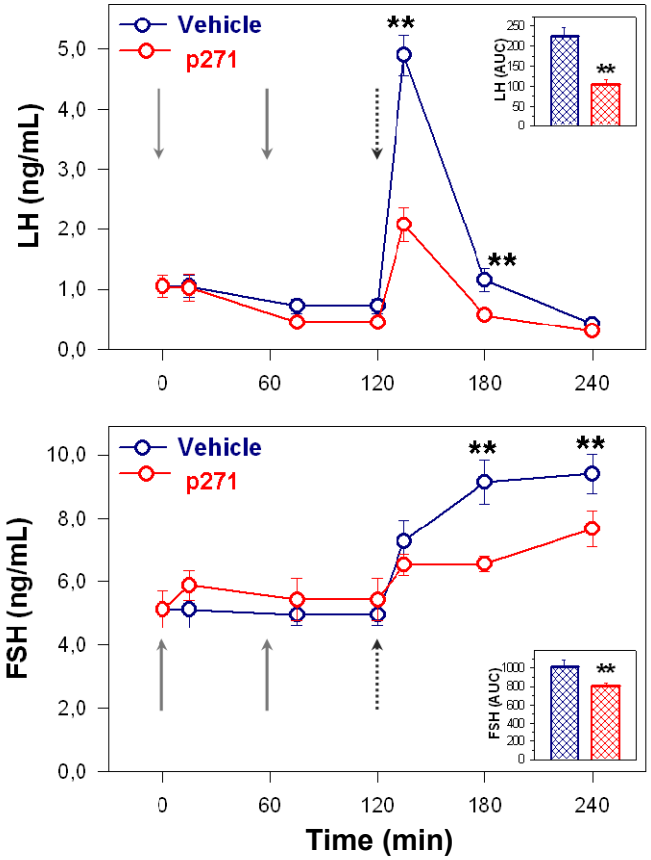

Figure 57. Effects of systemic kisspeptin antagonist on gonadotropin responses to icv Kp-10. LH and FSH secretory profiles are shown from adult male rats receiving three consecutive ip injections (5 nmol/each) of the antagonist of kisspeptin, peptide 271 (*denoted by arrows*); the last injection was associated with an icv bolus of Kp-10 (100 pmol). Integrated secretory responses following Kp-10 administration, calculated as area under the curve (AUC), are also depicted as bar graphs. ** P<0.01 vs. corresponding control group

3.3. Discussion

This research has been successful in identifying residues in kisspeptin critical to receptor binding and activation. It is clear that to bind to the receptor the pharmacophore involving Phe^{6}, Arg^{9} and Phe^{10} is crucially important; however Asn^{2} and Trp^{3} also appear to be needed. To activate gpr-54 the residues Tyr^{1} and Leu^{8} play a critical role, probably via stabilisation of the active conformation of the peptide and receptor. This suggests that residues in kp-10 required for receptor binding and for receptor activation overlap. Also, the conformation of the peptide is probably fundamental to peptide function as steric hindrance within the C-terminus disrupts receptor binding and activation.

The pleiotropic effects of kisspeptin make the gpr-54 receptor an attractive therapeutic target. Until now the emphasis has been on developing kisspeptin agonists as anti-metastatic agents (Clements et al., 2001; Orsini et al., 2007; Tomita et al., 2006). However, the development of kisspeptin antagonists would allow detailed analyses of the role of kisspeptin in the HPG axis and peripheral tissues. The ability to inhibit the actions of this system would facilitate examination of processes involved in regulation of puberty and the role of feedback mechanisms both *in vitro* and *in vivo*. Kisspeptin antagonists may also have useful therapeutic roles in pathophysiological conditions such as PCOS (where LH is over-expressed (Armeanu et al., 1992; Blank et al., 2006)), in precocious puberty to delay onset of sexual maturation (Navarro et al., 2004b), as putative novel contraceptive agents and for treating hormone-dependant diseases such as endometriosis and prostatic cancer.

To address the development of kisspeptin antagonists, a structure-activity study was undertaken of kisspeptin-10 analogues created by intuitive substitution of amino acid residues and by truncation of the peptide length. As the RFamide motif is highly conserved among species and members of the RFamide family, it was hypothesised that

this may be critical to receptor binding and activation. Therefore, the arginine and phenylalanine residues of the motif were substituted with D-Arg and Trp in both truncated and full-length analogues. Both peptides were unable to bind to or activate the receptor, confirming that this moiety is critical for peptide function. It has also been hypothesised that kp-10 is the smallest fragment able to bind and activate the receptor. To address this hypothesis, the receptor was truncated to five and seven amino acid fragments; both of which had severely reduced receptor binding, however the five amino acid fragments could still activate the receptor. Within the five residue fragments, if Leu^8 was inactivated by substitution with alanine or large amino acids, the peptide could no longer activate the receptor, suggesting Leu^8 of kp-10 may configure the receptor activation conformation. Furthermore, if Phe^6 was substituted then binding was ablated, suggesting a role for this residue in receptor binding. It has been suggested that the binding domain is in the C-terminus of kp-10 and the activation domain in the N-terminus (Ohtaki et al., 2001); however results within truncated peptides suggest that these functional domains overlap, with binding (Phe^6, Arg^9 and Phe^{10}) and activating residues (Leu^8) located throughout the length of the 10 amino acid sequence.

Results obtained with truncated peptides were confirmed with full-length 10 residue analogues, where substitution of Phe^6 with large D-amino acids dramatically reduced the peptides ability to bind to the receptor. This suggests that Phe^6 along with the amino acids of the RFamide moiety are critical for binding to the receptor, these observations have now been published by Orsini et al. who showed that these three residues (Phe^6, Arg^9 and Phe^{10}) form a binding pharmacophore that interacts with the receptor upon binding (Orsini et al., 2007). This research also showed that alanine substitution of Asn^2 and Trp^3 ablates receptor binding. This implicates Asn^2 and Trp^3 as additional residues that interact with the receptor upon binding. This is probably via formation of hydrogen bonds as asparagine and tryptophan are known to be capable of hydrogen bonding in other ligand-receptor scenarios (e.g. Trp^3 in GnRH with Asn^{98} of the receptor (Zhou et al., 1995)).

The role of Leu^8 in activating the receptor was also confirmed in studies with full-length kisspeptin-10 analogues, where substitution with either D-Leu or D-Trp inhibited receptor activation measured by production of inositol phosphate (IP) and intracellular calcium. These substitutions at position 8 also caused antagonism of the receptor, with D-Trp^8 being the most effective, suggesting that this residue is important for receptor activation. Tyr^1 was also shown to be involved in receptor activation in conjunction with Leu^8, as substitution of this residue with D-Trp or D-Ala increased the degree of antagonism. The need for flexibility around position 5 is suggested by amino acid residue changes made to Ser^5. When this side chain is altered by alanine substitution or introduction of a tight turn by substitution with proline occurs, the degree of antagonism is decreased. However, when Gly^5 or D-Ser^5 is introduced, there is greater flexibility of the centre of the peptide and this enhances antagonism, with Gly^5 being the more effective substitution. The need for Gly^5 and D-Trp^8 within the more potent antagonists was shown to be crucially important as substitution of either residue decreases the amount of antagonism at the receptor.

From the above research, a consensus sequence for the design of kisspeptin-10 antagonists was arrived at: X^1-N-W-N-X^5-F-G-X^8-R-F-NH_2 where X^1 = D-Tyr or D-Ala, X^5 = Gly or D-Ser and X^8 = D-Trp or D-Leu. The most potent amino acid changes for antagonism are present in peptide 234 where X^1 = D-Ala, X^5 = Gly and X^8 = D-Trp, however three other antagonists were also developed in peptides 230, 273 and 276.

Peptide 234 was then selected for *ex vivo* and *in vivo* studies. Since the role of kisspeptin within the HPG axis is thought to be the stimulation of GnRH release from the hypothalamus (Gottsch et al., 2006; Irwig et al., 2004) the ability of peptide 234 to inhibit kisspeptin-10 stimulation of firing in GnRH neurons was recorded in acutely prepared brain slices from the mouse. Peptide 234 was found to block kisspeptin-10 stimulation of GnRH neuron firing at 100nM, 10nM and 1nM. The low doses need to inhibit the firing of this neuron suggested that peptide 234 would also act as a potent antagonist *in vivo*.

The inhibitory effect of peptide 234 on the firing of GnRH neurons suggested that this peptide would also be able to inhibit the pulsatile GnRH secretion from the median eminence. It was therefore examined whether peptide 234 administered directly to the stalk-median eminence region of pubertal female rhesus monkeys would suppress the amplitude or frequency of GnRH pulsatility. The suppression of GnRH pulse amplitude seen during peptide 234 infusion provides the first direct evidence that signalling from kisspeptin neurons in the hypothalamus is required for GnRH pulsatile secretion. This had previously been suggested through observations that kisspeptin-54 pulses coincide with GnRH pulses 75% of the time (Keen et al., 2008). However, as patients with mutations in gpr-54 have been shown to retain some GnRH pulsatility (indirectly through recording LH pulses) within the hypothalamus with normal frequency (de Roux et al., 2003; Seminara et al., 2003; Tenenbaum-Rakover et al., 2006), it has been questioned whether inputs from the kisspeptin neuron are responsible for the development of GnRH pulsatility or if kisspeptin neurons regulate GnRH pulse amplitude. The dampening of spontaneous GnRH pulses in the monkey model suggests that kisspeptin is required for the amplitude of GnRH pulsatile secretion. However, it remains unclear whether frequency is also affected. Work with peptide 234 within Kevin O'Byrne's laboratory at Kings College London, has suggested the antagonist can delay pulse frequency, however, further work is still to be completed to confirm these findings.

Peptide 234 also blocked kisspeptin-10 stimulation of LH secretion in adult male rats and mice presumably by blocking kisspeptin-10 effects on GnRH secretion. In rats the inhibition was incomplete at 1nmol peptide 234 suggesting that the dose may be sub-optimal in relation to the size of the rats used. Interestingly, the same dose completely inhibited the kisspeptin-10 stimulation of LH secretion in mice as did 5nmol and 15nmol. Ovariectomised sheep were then used to examine if the inhibitory effect was due to a reduction in LH pulse amplitude as with GnRH. In these animals, LH pulse amplitude was markedly reduced by administration of peptide 234. This is consistent with the finding that peptide 234 inhibited GnRH neuron firing rate in mice and reduced

GnRH pulse amplitude in rhesus monkeys. These effects of peptide 234 appear to be specific to the regulation of gonadotropins since the antagonist had no effect on either prolactin or cortisol secretion in ovariectomised ewes.

One unexpected result from this research was the lack of effect that peptide 234 had on basal levels of GnRH and LH. This suggests that kisspeptin is responsible for the pulsatile secretion of GnRH but other factors underlie the basal secretion. This hypothesis is supported by the fact that peptide 234 did not lower the basal firing rate of the mouse GnRH neurons and had no effect on basal GnRH secretion in the female rhesus monkey. This was also the case in studies on LH secretion, where peptide 234 failed to inhibit LH secretion below intact basal levels in rodents and ewes. Further confirmation is still needed to establish that kisspeptin is not responsible for the basal secretion of LH in females as well as males. This would be an important discovery as the ability to inhibit the ovulatory LH surge but not basal LH in females might provide a novel contraceptive that does not ablate endogenous estrogen yet blocks ovulation. (Lapatto et al., 2007). To investigate this further, peptide 234 was administered to cyclic female rats on the morning of estrus and was shown to completely ablate the expected pre-ovulatory LH and FSH surges compared to controls. However, the basal levels of LH and FSH were not affected, suggesting that kisspeptin does not regulate basal secretion of GnRH in female rodents as well as males. These observations directly implicate kisspeptin in the production of the LH surge and mediation of positive feedback mechanism, and provide further evidence for the use of peptide 234 as a novel contraceptive, which can ablate the LH surge but will not affect basal levels of gonadotropins and steroids.

In male mice peptide 234 blocked the increase in LH that ensues following castration. A similar result was observed in castrated rats but the inhibition was less pronounced and delayed probably due to the lower dosage per g/bodyweight employed and the consequent requirement for an accumulation of peptide 234 in the brain over the 3 injections. These findings suggest that KiSS-1 neurons are a target for steroid hormone

action and involved in the negative steroid feedback mechanism within the hypothalamus, relaying effects to GnRH neurons. This had previously been suggested in view of increased *KiSS-1* gene expression in the arcuate nucleus in gonadectomised male and female rats (Gottsch et al., 2006; Irwig et al., 2004) which was hypothesised to be due to a negative feedback mechanism (Smith et al., 2005b). However, changes in mRNA do not always reflect functional changes in the biosynthesis and secretion of kisspeptin within the arcuate nucleus. The studies with the novel kisspeptin antagonist provide direct evidence for kisspeptin modulation of the GnRH neuron in negative feedback and emphasize the value of kisspeptin antagonists for elucidating physiological mechanisms.

The inhibition of GnRH and LH pulses in pubertal female rhesus monkeys and OVX ewes by peptide 234 suggests a role for kisspeptin in the initiation of puberty. The demonstration that kisspeptin-10 administration advanced the age of vaginal opening in female rats has previously implicated kisspeptin in the onset of puberty (Navarro et al., 2004b). Further studies with peptide 234 in peri-pubertal female rats over 7 days were conducted to confirm a role for kisspeptin in puberty onset. Administration of peptide 234 to these rats delayed vaginal opening and reduced ovarian and uterine weights, directly implicating kisspeptin in puberty onset.

However, as peptide 234 had to be delivered icv in all of the above experiments, this would not be suitable for taking forward into human trials on precocious puberty until systemic efficiency can be demonstrated. Therefore, a penetratin tag was added to peptide 234 to increase permeability at the blood-brain barrier and potentially extend its half-life. This modified peptide inhibited LH and FSH secretion in the rat when 5nmol were delivered by ip injection, a method that would be better translated into clinical studies.

As well as the development of gpr-54 antagonists, the other aim of the structure-activity studies was to identify residues within kp-10 important for binding to gpr-54 or for

receptor activation. This would then facilitate interpretation of the secondary structure of kp-10 and give an insight into the types of bonds formed within the binding interactions. This information would facilitate the receptor binding pocket being modeled. From the structure activity study of kp-10 within this chapter, it was shown that Phe^{6}, Arg^{9} and Phe^{10} were critical for kp-10 to bind to gpr-54 and this has subsequently been confirmed by another group within the field (Orsini et al., 2007). These residues can therefore be investigated using the NMR structures for kp-10 (Lee et al., 2009; Orsini et al., 2007). The first NMR model was a helical structure and within this model the phenyl rings of Phe^{6} and Phe^{10} are stacked on top of each other with the positive charge of Arg^{9} in the middle. The second model suggests a tight turn structure for kp-10, but again the positioning of these three residues supports a similar pharmacophore with the two stacked rings flanking the positively charge arginine. Both models therefore suggest that two types of bonds may be formed between the ligand and receptor, firstly, ionic bonds may be formed between the positive charge of Arg^{9} and negatively charged amino acids within the receptor binding pocket and secondly, stacking interactions may occur between the two phenyl rings of Phe^{6} and Phe^{10} and any aromatic residues within the receptor binding pocket. However, the ionic bonds may be more important than the stacking interactions as this bond is stronger than the van der waals forces involved in the stacking interactions. This possibility is consistent with the structure-activity data, where moving the positioning of the side chains of $Phe^{6, 10}$ ablates the binding to the receptor signifying that the side chain position is critical, whereas moving the side chain of Arg^{9} only slightly reduces the binding suggesting that the charge is the important factor.

Now that the binding pharmacophore for kp-10 is known, this can be used along with further structure-activity studies to examine the binding pocket within gpr-54. This binding pocket is likely to be in the following area: juxtamembrane, within a structure formed by the extracellular loops and cell surface regions of the transmembrane domains. The position of the pocket can be addressed using two methods, mutation of specific amino acids within gpr-54 or by constructing a homology model based on the

tertiary structure of other GPCRs. Receptor mutations are useful to provide information on the specific residues involved in the binding of kp-10 and the types of inter-molecular bonds involved. However, this is a time consuming process and would benefit from a homology model of gpr-54. A homology model of the receptor could be based on the primary sequence of gpr-54 and a template of the likely tertiary structure taken from a related protein, such as the β2 –adrenergic receptor (a class A GPCR). The results are more accurate if the sequence alignment between the two receptors is > 50%, with the most sequence divergence within the loop regions. For less conserved areas, constraints can be applied such as internal co-ordinates and dihedral angles. The model may then be used to virtually dock the ligand to access the most likely location of the binding pocket.

A ligand docking homology model has been prepared for gpr-54 by Zhi-liang Lu (MRC HRSU, Edinburgh) but is still in its infancy (Fig. 58). However from the model it is possible to see that the predicted binding pocket lies within the hypothesized site interacting with the extracellular loops (ECL) and the cell surface regions of the transmembrane (TM) domains. Specific binding residues within the receptor are still to be deciphered but the regions for these interactions can be predicted. The kp-10 binding pharmacophore seems to interact at two sites, firstly $Phe^{6, 10}$ are situated close to the cell surface region of TM6/7 and Arg^{9} is in the vicinity of TM2/3. These are the only residues of kp-10 which project into the TM domains, which may provide a protective environment for binding to occur and suggests that the main gpr-54 binding pocket is close to the core of the peptide. Other residues were also shown to be involved in binding within this chapter, mainly Asn^{2} and Trp^{3}. These both lie close to the ECLs of gpr54, specifically ECL1, although Trp^{3} may also interact with the N-terminal region. The two residues not examined in this thesis, Asn^{4} and Gly^{7} are also situated close to the ECL regions with Asn^{4} near to ECL2 and Gly^{7} in the vicinity of ECL3. This suggests that some residues may be involved in positioning of the peptide to allow the binding pharmacophore to interact with the TM domains. However, as modeling is a complex process the exact interactions are still to be examined.

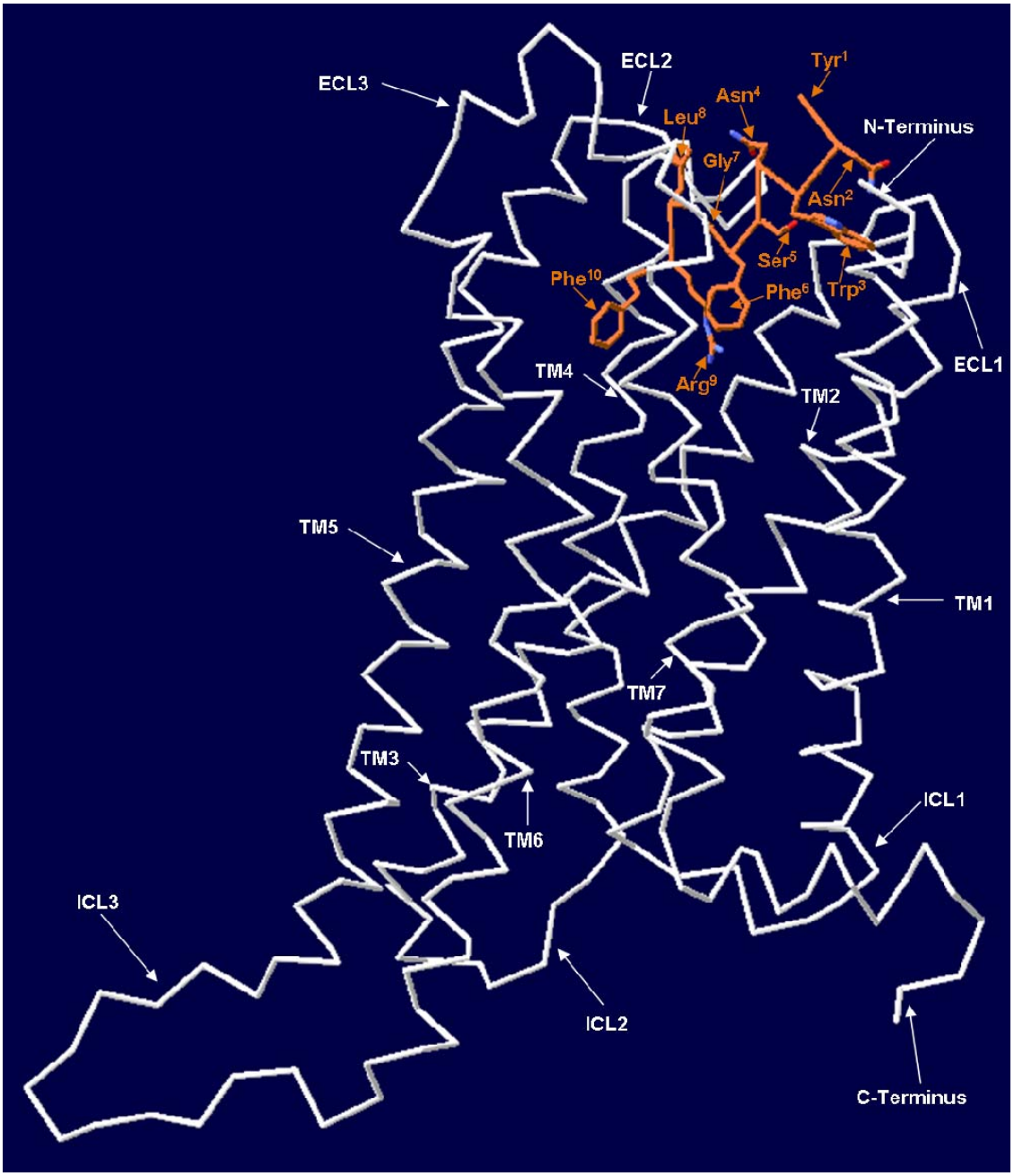

Figure 58. Homology model of gpr-54 showing the docking of kp-10. Homology model showing the predicted structure for gpr-54 (white). Within this model kp-10 (orange) has been virtually docked to gpr-54 to assess the possible binding interaction. This model was designed by Dr Z Lu at the MRC HRSU in Edinburgh.

In conclusion, this part of my research has been successful in elucidating the residues in kisspeptin-10 important for receptor binding and activation, with the creation of four potent antagonists. One of these antagonists has been characterised extensively *in vivo* and has been shown to inhibit both GnRH neuron firing and pulstatile secretion with subsequent inhibition of LH pulse amplitude, supporting a role for kisspeptin in modulation of the HPG axis. This antagonist has also provided direct evidence of a role for kisspeptin in modulating both negative and positive steroid feedback from the gonads in males, the production of the LH surge in females and regulation of the onset of puberty in female rats.

Chapter Four

Intracellular signalling pathways activated by kisspeptin-10 and gpr-54

4.0. Abstract

Kisspeptin and gpr-54 have been placed at the centre of the reproductive axis since it was discovered that kisspeptin neurons can directly innervate GnRH neurons, on which the gpr-54 receptor has been located. Kisspeptin neurons have since been implicated in relaying multiple environmental, metabolic and steroidal cues to GnRH neurons to control GnRH secretion and hence reproductive tissue function. Kisspeptin has also been implicated as a key regulator within the placenta to control the invasion of trophoblast cells into the maternal uterine deciduum. The major focus to date has been to delineate physiological actions of the kisspeptin system using *in vivo* models, with less attention focussed on the molecular mechanisms effecting these kisspeptin/gpr-54 actions. Therefore, in the following chapter, the molecular signalling pathways utilised by kp-10 upon activation of gpr-54 have been studied, firstly in a transfected CHO cell line. Gpr-54-activated signalling pathways found in this cell line were then tested in mouse embryonic GnRH neuronal cells (GT_{1-7}), human breast cancer cells (MCF-7) and a human first trimester extravillious trophoblast cell line (HTR8SVneo). In CHO cells, kisspeptin stimulated phosphorylation of members of the MAPK pathway occurred, with a robust activation of ERK1/2 via an EGFR transactivation pathway. ERK1/2 phosphorylation was also evident in the neuronal, cancer and trophoblast cell lines. Also within three cell types (CHO/gpr-54, GT_{1-7} and HTR8SVneo), phosphorylation of NFκB occurred via PKC. In the CHO and trophoblast cell line, the most robust signal was the inhibition of GSK3β by phosphorylation, causing release of β-catenin into the cytoplasm. In both cell types, GSK3β inactivation occurred by Ser^{9} phosphorylation, mediated by the ERK1/2 target, p90rsk, PKC and the PI(3)K/Akt pathways. GSK3β inhibition and release of β-catenin was further amplified by a feedback loop involving FAK phosphorylation at Tyr^{925}. This facilitates binding of the Grb-SOS complex to then activate RAS, further phosphorylating ERK1/2 and p90rsk. Elevated levels of

cytoplasmic β-catenin may interact with cadherins to inhibit cell movement, suggesting a possible mechanism for kp-10-mediated inhibition of trophoblast migration. In summary, the signalling pathways within trophoblast cells and GnRH neurons have been investigated and show cell-specific activation of signalling pathways.

4.1. Introduction

Kisspeptin/gpr-54 signalling has emerged as a linchpin in the neuroendocrine regulation of reproduction (Popa et al., 2008), since it was discovered that kisspeptin-producing neurons directly innervate GnRH neurons and that kisspeptins are potent secretagogues for GnRH and LH (Gottsch et al., 2004; Irwig et al., 2004; Popa et al., 2008). Moreover, kisspeptin neurons have been implicated as conduits that relay humoral and environmental signals to GnRH neurons (Castellano et al., 2005; Estrada et al., 2006; Greives et al., 2006; Revel et al., 2006; Smith et al., 2005b). Kisspeptins were originally identified as inhibitors of metastasis and trophoblast invasion (Bilban et al., 2004; Kotani et al., 2001). However, the intracellular signalling events by which kisspeptin modulates cell migration are poorly understood.

In the GnRH neuron, calcium influx through sodium-dependant cationic channels in the plasma membrane and from intracellular stores is needed to stimulate secretion of GnRH in response to kisspeptin (Liu et al., 2008; Zhang et al., 2008). Kisspeptin-10 also inhibits trophoblast cell migration and NFκB signalling has been implicated in this process via regulation of matrix metalloproteinase-9 (MMP-9) gene expression (Yan et al., 2001). Finally some mechanisms for kisspeptins effects on metastasis have been identified such as desensitization of CXCR4 responses to SP-1 to decrease calcium release and Akt/PKB phosphorylation, in turn blocking chemotaxis (Navenot et al., 2005). In epithelial-like CHO cells it has also been shown that the receptor couples to the $G_{q/11}$ class of G-proteins to classically activate phospholipase C which hydrolyses

phosphatidyl bisphosphate (PIP2) in the cell membrane to diacyl glycerol (DAG) and inositol triphosphate (IP_3), which activate PKC and modulate intracellular calcium, respectively. This leads to phosphorylation of ERK1/2 and p38MAPK, cellular reorganisation of stress fibres and focal adhesion complexes. Effects on the cytoskeleton and cell adhesion molecules are thought to be important for kisspeptin inhibition of cancer cell metastasis and trophoblast invasion (Hori et al., 2001; Kotani et al., 2001). However, a detailed signalling study of the mechanisms downstream of gpr-54 activation especially those relating to cell migration are yet to be undertaken.

In order to examine the signalling pathways induced by kp-10, a variety of cell types were utilised to examine the likely existence of differential signalling between tissues such as neurons and the placenta and between cell types such as epithelial versus neuronal cells. In this study, these differences were examined using an epithelial CHO cell model, malignant epithelial MCF-7 breast cancer cells, epithelial Ishikawa uterine cancer cells, GnRH neuronal cells (GT_{1-7}), B35 neuroblastoma cells, GH3 neuroendocrine somatolactotroph cells, choriocarcinoma trophoblast cells, and HTR8SVneo immortalised trophoblast cells (Table 9). Some of these cells were transfected with gpr-54 expression construct in order to highlight any differences between endogenous versus transfected receptor expression.

	Transfected receptor	Endogenous receptor
Epithelial	CHO (Chinese Hamster Ovary)	MCF-7 (Breast cancer cells) Ishikawa (Uterine cancer cells)
Neuronal	GT_{1-7} (GnRH neuronal cells) B35 (neuroblastoma cells)	B35 (neuroblastoma cells) GH3 (somatotrope cells)
Trophoblast		JEG-3 (Choriocarcinoma cells) JAR (Choriocarcinoma cells) BeWo (Choriocarcinoma cells) HTR8SVneo (immortalised trophoblast cells)

Table 9. Cell types utilised to analyse kp-10 mediated signalling. Table showing origin and phenotype for each cell line and if the receptor is transfected or endogenous to the cells.

This approach should also allow examination of cell type specific signalling, which may account for the wide variety of processes mediated by kisspeptin, for example, GnRH secretion may involve a different signalling network to kisspeptin-mediated inhibition of cell migration. These pathways may be completely distinct from one another or may involve common components to regulate gene expression, remodel the cytoskeleton, or regulate the secretion of hormones in different tissues (Fig. 59). Overall, cell type specific signalling is important to allow a single ligand to have a variety of actions throughout the body.

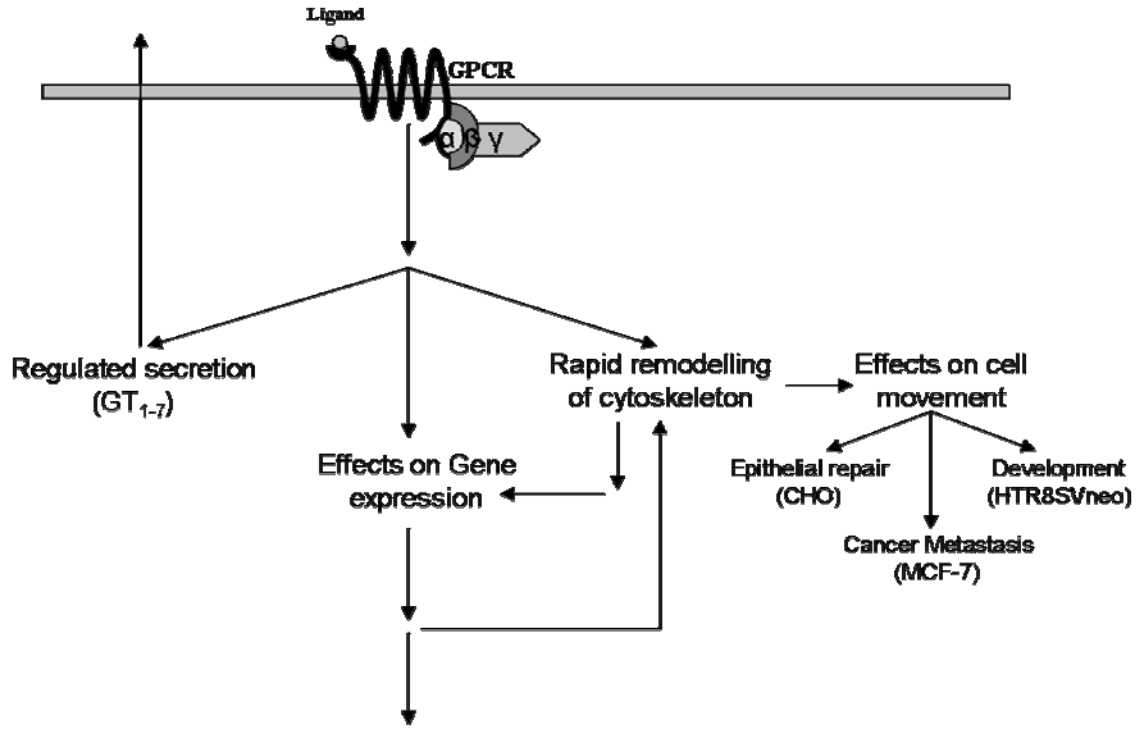

Figure 59. Possible cellular processes mediated by gpr-54 signalling. Diagram showing possible processes within the cell that could be mediated by kp-10 in different cells types (shown in brackets). These effects may be distinct such as hormone secretion or may influence each other such as gene expression and cytoskeleton remodelling.

This chapter reports on the elucidation of intracellular signalling pathways activated by kisspeptin-10 at gpr-54 potentially involved in the inhibition of cell migration and GnRH secretion. The results show that kisspeptin-10 can activate the NF-kappa B, focal adhesion kinase (FAK), GSK3β and mitogen-activated protein kinase (MAPK) signalling pathways and inhibitors have been used to further elucidate these signalling mechanisms in CHO/gpr-54, GnRH neuronal, trophoblast and cancer cell lines.

4.2. Results

4.2.1. Kisspeptin-10 signals via MAPK, NFκB and focal adhesion pathways in CHO cells stably expressing the human gpr-54

As kp-10 is known to be the minimal peptide sequence needed for activation of gpr-54, the model cell line, CHO/gpr-54, was tested for receptor binding and activation. Kp-10 bound to the human gpr-54 displaced ^{125}I-kisspeptin-10 with an IC_{50} of $7.7x10^{-9}$M. This confirmed that the cell line expressed the receptor at the cell surface. Kisspeptin has previously been shown to activate $G_{q/11}$ and stimulate IP_3 production and calcium mobilisation through gpr-54 (Hori et al., 2001; Kotani et al., 2001). Therefore inositol phosphate and intracellular calcium activation were examined. In CHO/gpr-54, kp-10 robustly stimulated both IP production and calcium mobilization with EC_{50s} of $3.8x10^{-9}$M and $6x10^{-8}$M, respectively (Fig. 60). Kp-10 could also activate an SRE-luciferase reporter gene in another stable cell line, the mouse pituitary cell line $L\beta T_2$ stably expressing the mouse gpr-54. This confirms that gpr-54 and kp-10 activated the $G_{q/11}$ class of g-proteins. Untransfected CHO cells gave no IP response when stimulated with kp-10 (Fig. 60)

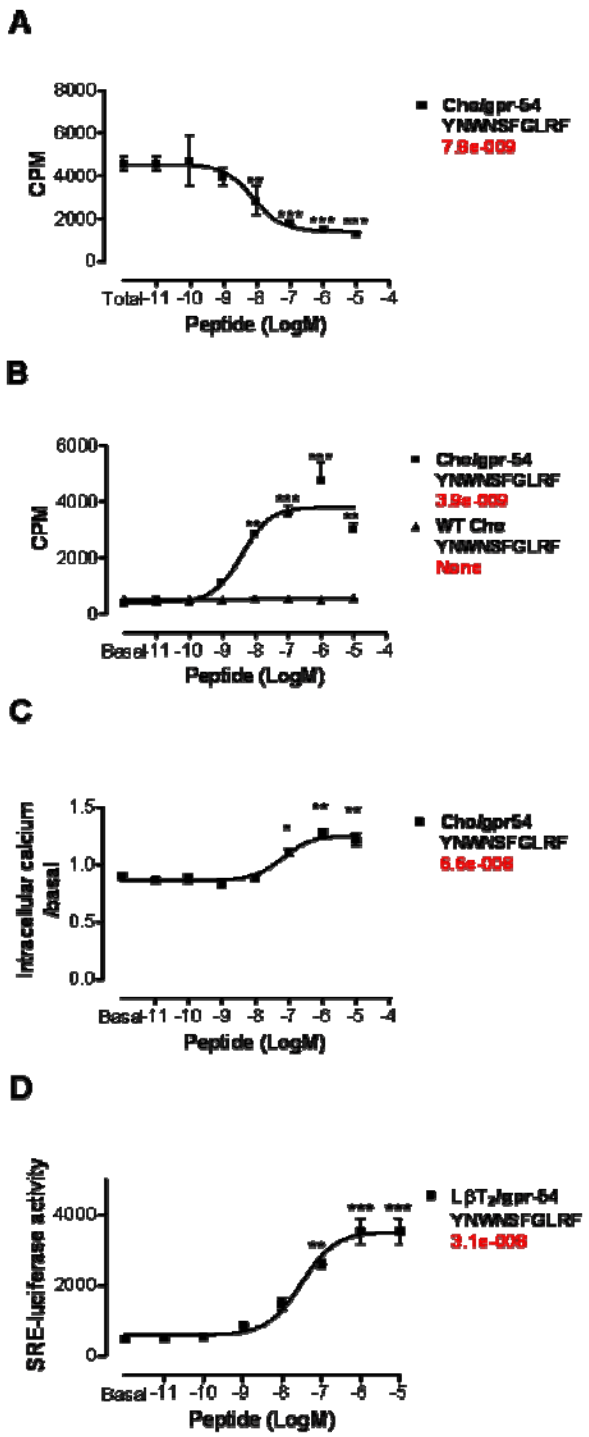

Figure 60. Kisspeptin binds and activates CHO cells stably expressing human gpr-54. (A) Kisspeptin can bind gpr-54 with an affinity of 7.8nM in these cells (n=10). (B, C) Kisspeptin can activate IP (n=12) and intracellular calcium release (n=5) with an EC_{50} of 3.9nM and 66nM, respectively. (D) In $L\beta T_2$/gpr-54 cells, kp-10 can also increase a SRE-luc reporter gene (n=3).

As phosphorylation of ERK1/2 had already been demonstrated (Hori et al., 2001; Kotani et al., 2001), studies firstly concentrated on the MAPK signalling pathway. Kp-10 stimulated ERK1/2 phosphorylation in a dose and time dependant manner. ERK1/2 phosphorylation increased significantly with dose and 10 and 100nM gave a maximal stimulation after 10 minutes (Fig 61). Thereafter, 10nM kp-10 was used as the standard dose for further studies. 10nM kp-10 also stimulated a significant phosphorylation of c-Jun NH_2-terminal protein kinase 1 (JNK1) and p38MAPK with a maximal response for both at 10 minutes (Fig. 62). As ERK1/2 gave a robust phosphorylation inhibitors were used to determine the intracellular pathway responsible for this phosphorylation. A $G_{q/11}$ inhibitor and a MEK inhibitor, completely blocked kp-10 stimulation of ERK1/2 phosphorylation as did inhibitors of Src, PI(3)K, Akt, p90rsk and GSK3β (Fig. 61). This suggested that Kp-10 activated the classical RAS-Raf-1-MEK-ERK1/2 pathway possibly via EGFR receptor transactivation.

Next, it was investigated if kp-10 activates proteins associated with cell migration. This involved the investigation of two pathways, focal adhesion complex associated signalling, as modulation of focal adhesion complexes has previously been reported (Kotani et al., 2001) and the NFκB pathway, also previously implicated in the context of modification of the extracellular matrix (Yan et al., 2001). However, the details of the signalling mechanisms involved have so far not been elucidated. 10nM kp-10 significantly stimulated phosphorylation of focal adhesion kinase (FAK) at 10-30 minutes (Fig.63). This phosphorylation was significantly repressed by inhibitors of $G_{q/11}$, Src, PI(3)K, MEK, p90rsk, GSK3β and Akt/PKB (Fig. 63). This suggests FAK is phosphorylated via an ERK1/2-p90rsk/Akt-GSK3β pathway. NFκB was also significantly phosphorylated upon stimulation with 10nM kp-10 with a maximal stimulation at 30 minutes. This activation was suppressed by inhibitors of $G_{q/11}$ and PKC (Fig. 64). The inhibitor of NFκB (IκBα) was also examined and was dephosphorylated at Ser^{32} throughout the 60 minute stimulation by 10nM kp-10 (Fig. 64). This seems unusual as phosphorylation of IκBα at Ser^{32} is required to enable release of NFκB into the nucleus. Dephosphorylation at this residue should restrict

NFκB release. However, as the IκBα signal is weak the results are difficult to interpret; therefore immunoprecipitation experiments may need to be performed to confirm these results.

4.2.2. Kisspeptin-10 modulates GSK3β and β-catenin pathways in CHO cells stably expressing human gpr-54

Next it was decided to investigate if other pathways normally associated with migration were activated in these cells. One such pathway is the adherens junction pathway, where β-catenin can associate with cadherins to form adherens junctions, thus inhibiting cell movement. The main signalling pathway associated with β-catenin involves Src and PI(3)K activation of Akt which can inhibit glycogen synthase kinase 3β (GSK3β) by phosphorylating Ser^9, allowing β-catenin to be released from a complex with GSK3β (Dominguez and Green, 2001). Akt then phosphorylates β-catenin to cause its translocation to the nucleus (Fang et al., 2007). However, a second mechanism for β-catenin release involves ERK1/2 activation of p90rsk which also inhibits GSK3β, allowing β-catenin to associate with cadherins (Torres et al., 1999). Therefore investigations were performed to examine the effect of kp-10 on these proteins. 10nM kp-10 stimulation significantly induced phosphorylation of Src, GSK3β (Ser^9) and increased cytoplasmic levels of β-catenin over 60 minutes (Fig. 65). Inhibitors were again used to delineate the mechanisms involved. GSK3β phosphorylation was blocked by inhibitors of $G_{q/11}$, Src, PI(3)K, Akt, PKC, p90rsk and GSK3β whereas β-catenin was inhibited by $G_{q/11}$, PI(3)K, Akt, PKC, MEK and p90rsk inhibitors (Fig. 65). The p90rsk inhibitor decreased β-catenin levels to below basal, and this is probably due to pools of β-catenin with GSK3β that cannot be phosphorylated at Ser^9 due to this residue being masked within the β-catenin destruction complex.

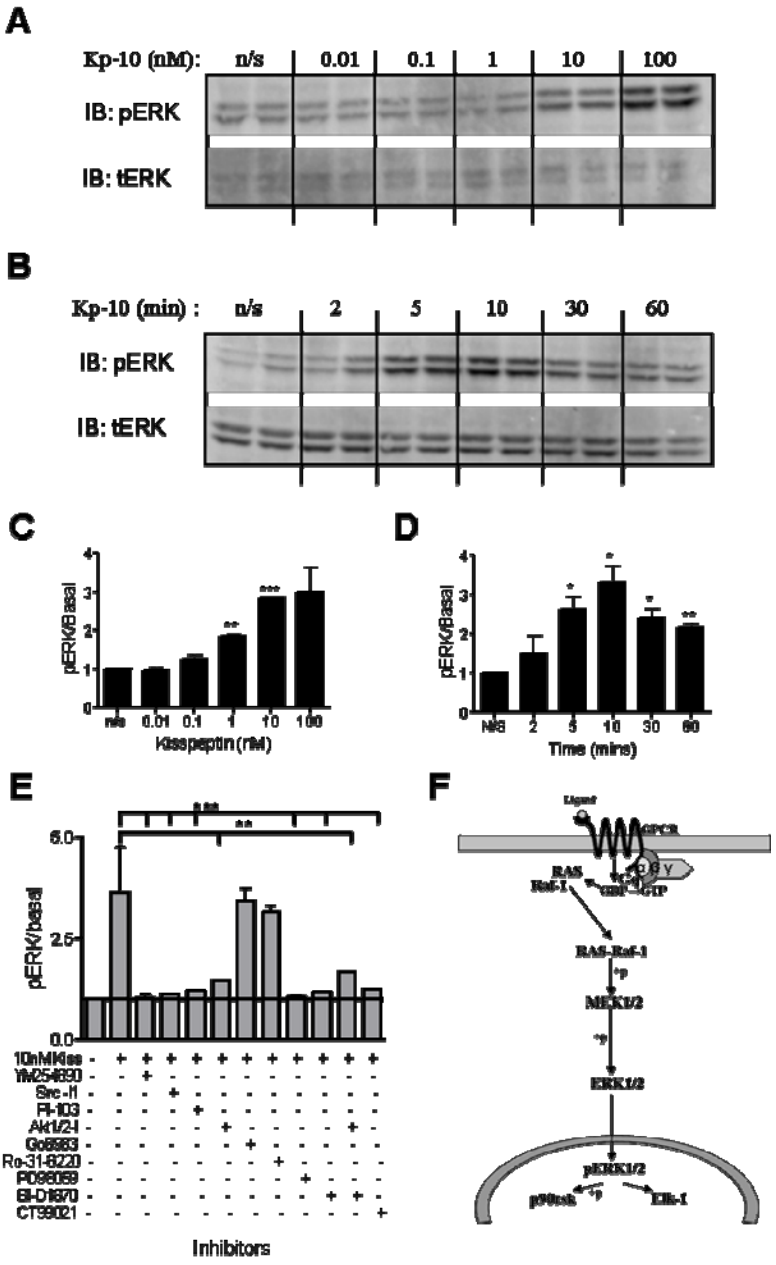

Figure 61. Kisspeptin activates ERK1/2 via the classical pathway. (A) Kisspeptin phosphorylates ERK1/2 in a dose dependant manner at 10 minutes (n=4). (B) 10nM Kisspeptin phosphorylates ERK1/2 in a time dependant manner peaking at 10 minutes (n=4). (C, D) Quantification of dose and time dependant ERK1/2 activation showing significant phosphorylation from 1nM and between 5 and 60 minutes compared to total ERK. (E) Kisspeptin-dependant ERK1/2 phosphorylation is significantly decreased by 100nM YM254890 ($G_{q/11}$), 1uM Src-I1 (Src), 1uM PI-103 (PI(3)K), 1μM Go6983 (PKC α, β, γ, δ, μ, ζ), 1μM Ro-31-8220 (PKC α, β, γ, ε),20μM PD98059 (MEK), BI-D1870 (p90rsk) and CT99021 (GSK3β) inhibitors (n=3) and (F) diagram of classical ERK signaling pathway.

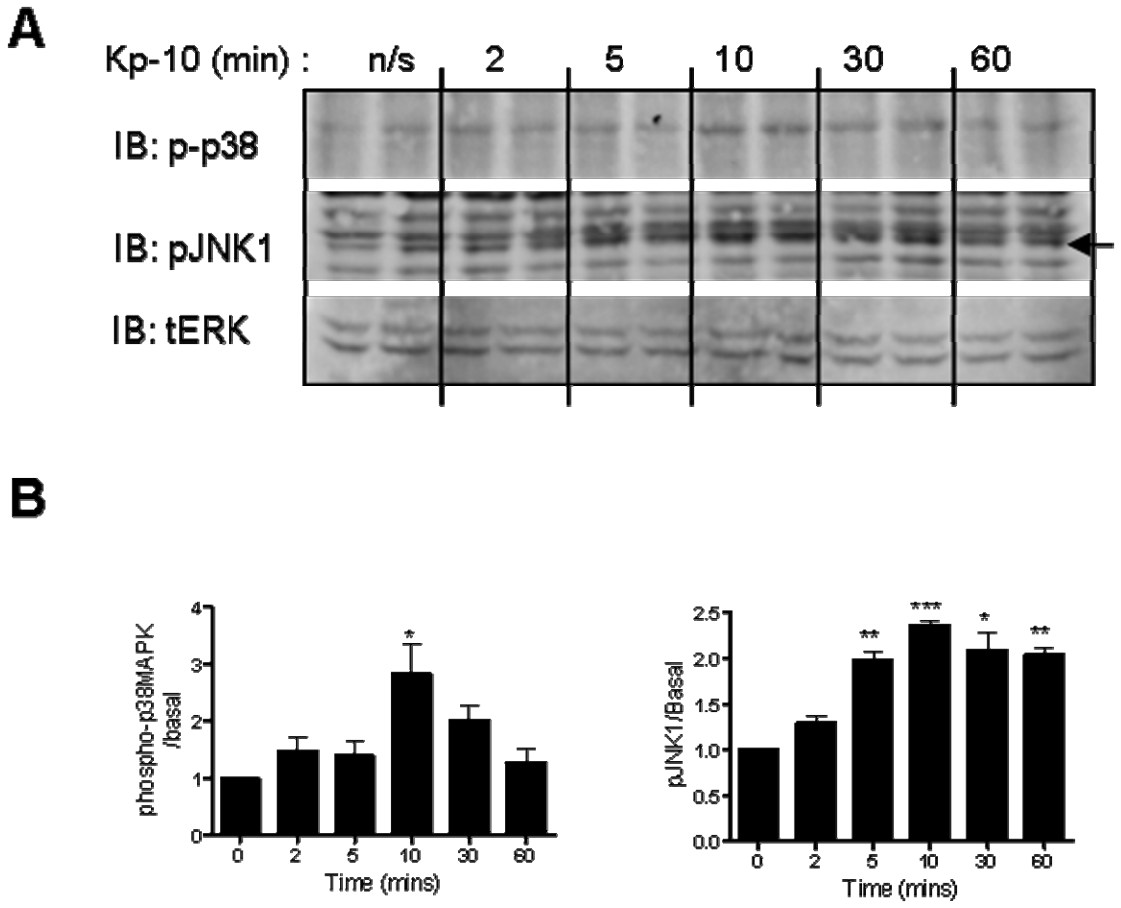

Figure 62. Kisspeptin activates other members of the MAPK pathway. (A) Kisspeptin phosphorylates p38MAPK and JNK1 (n=3). Total ERK1/2 was used as a loading control. (B) Quantification for phosphorylation of p38MAPK with an increase at 10minutes and JNK1, which is significantly increased at 5-60 minutes.

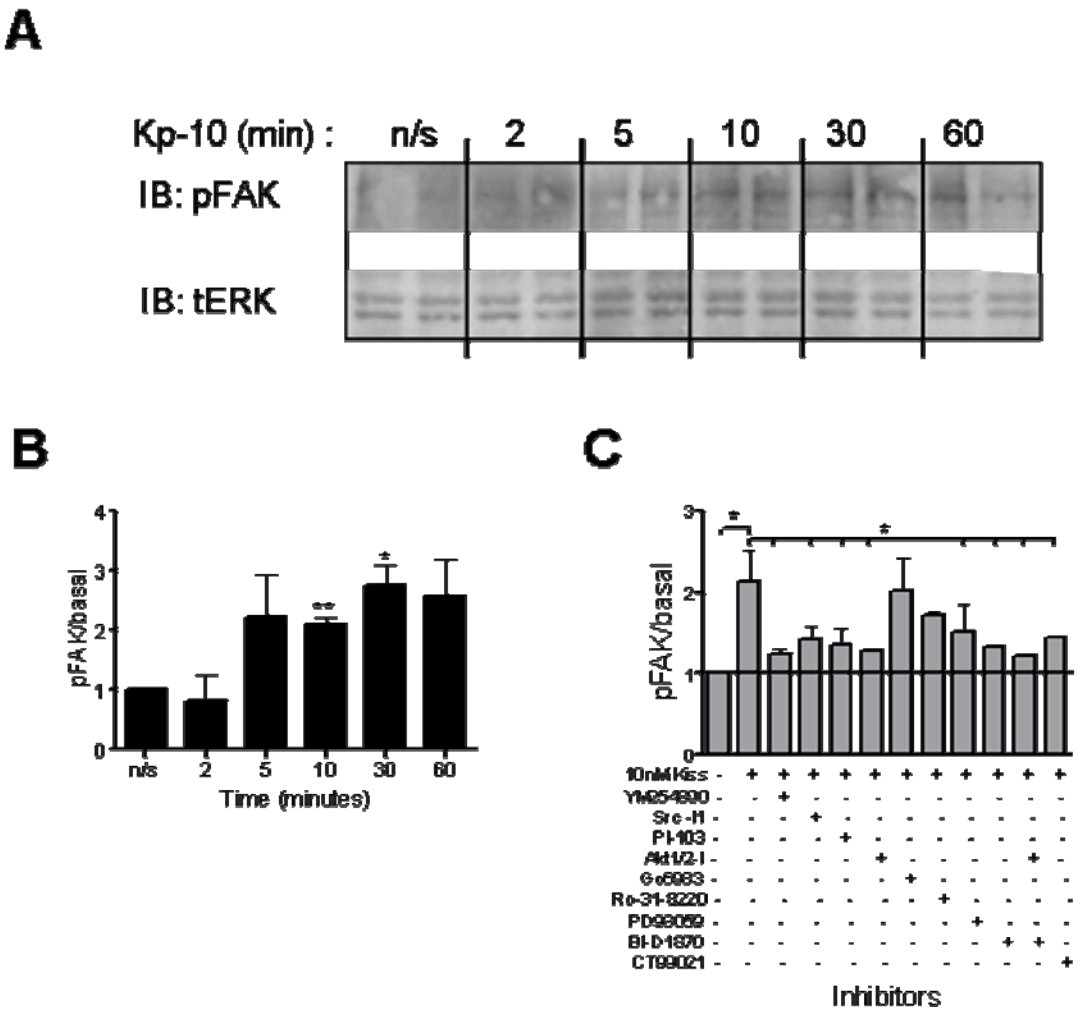

Figure 63. Kisspeptin phosphorylates focal adhesion kinase (FAK) in CHO/gpr-54 cells. (A) Kisspeptin phosphorylates FAK from 5-60 minutes (n=3). Total ERK1/2 is used as a loading control. (B) Quantification of FAK phosphorylation compared to loading control shows significant increases from 10-30 minutes. (C) This increase at 30 minutes is significantly decreased by 100nM YM254890 ($G_{q/11}$), 1uM Src-I1 (Src), 1uM PI-103 (PI(3)K), 1μM Akt-Inhibitor 1/2 (Akt/PKB), 20μM PD98059 (MEK), 1μM BI-D1870 (p90rsk) and 1μM CT99021 (GSK3β) inhibitors (n=4).

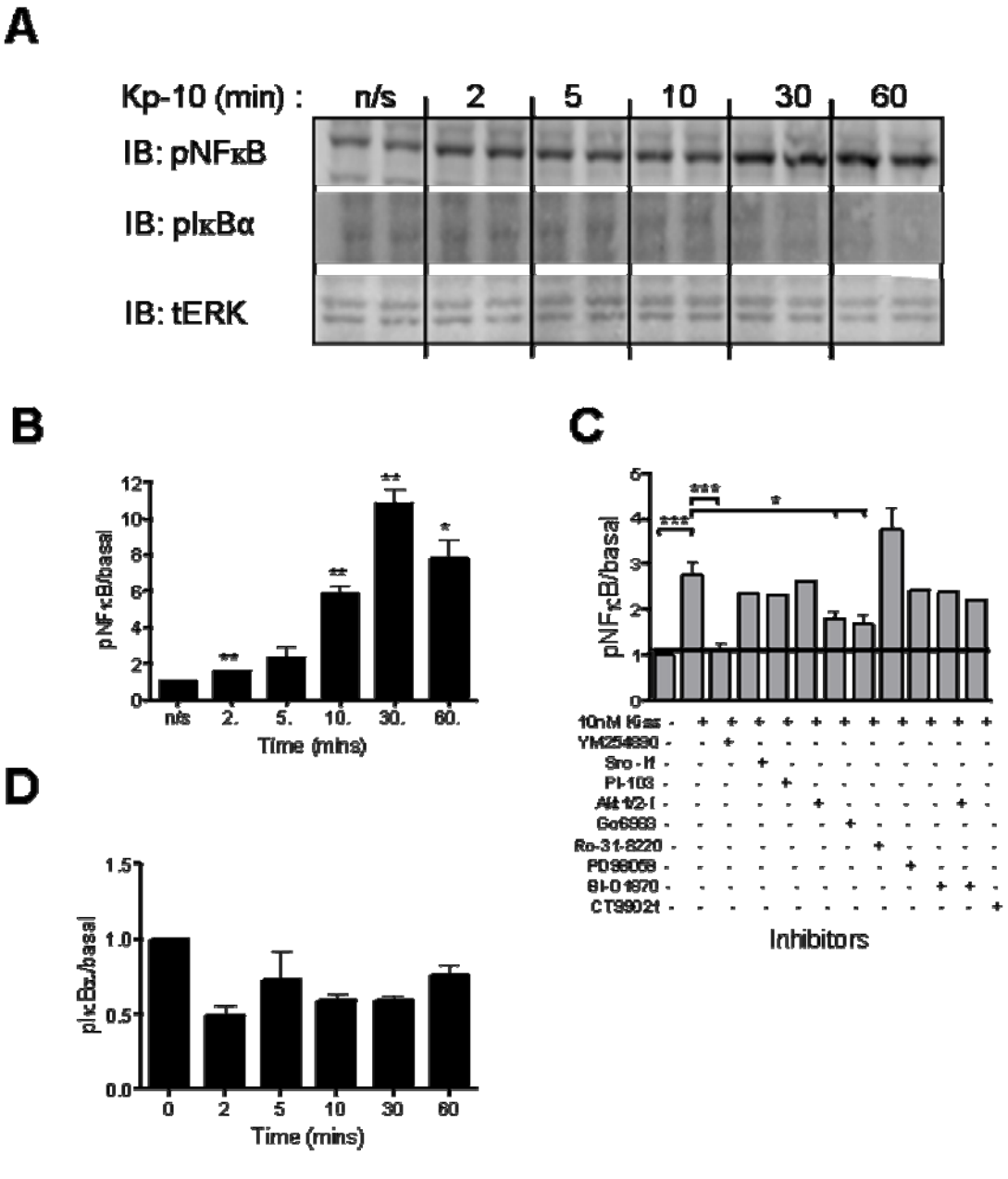

Figure 64. Kisspeptin activates phosphorylation of NFκB in CHO/gpr-54 cells. (A) Kisspeptin phosphorylates NFκB (n=3) but de-phosphorylates IκBα (n=2). Total ERK1/2 was used as a loading control. (B) Quantification of NFκB phosphorylation by kisspeptin reaching significance from 10-60 minutes. (C) This phosphorylation is blocked by 100nM YM254890 ($G_{q/11}$), 1μM Go6983 (PKC α, β, γ, δ, μ, ζ) and 1μM Ro-31-8220 (PKC α, β, γ, ε) inhibitors (n=4). (D) IκBα, an inhibitor of NFκB was dephosphorylated at Ser^{32} throughout the 60 minutes examined.

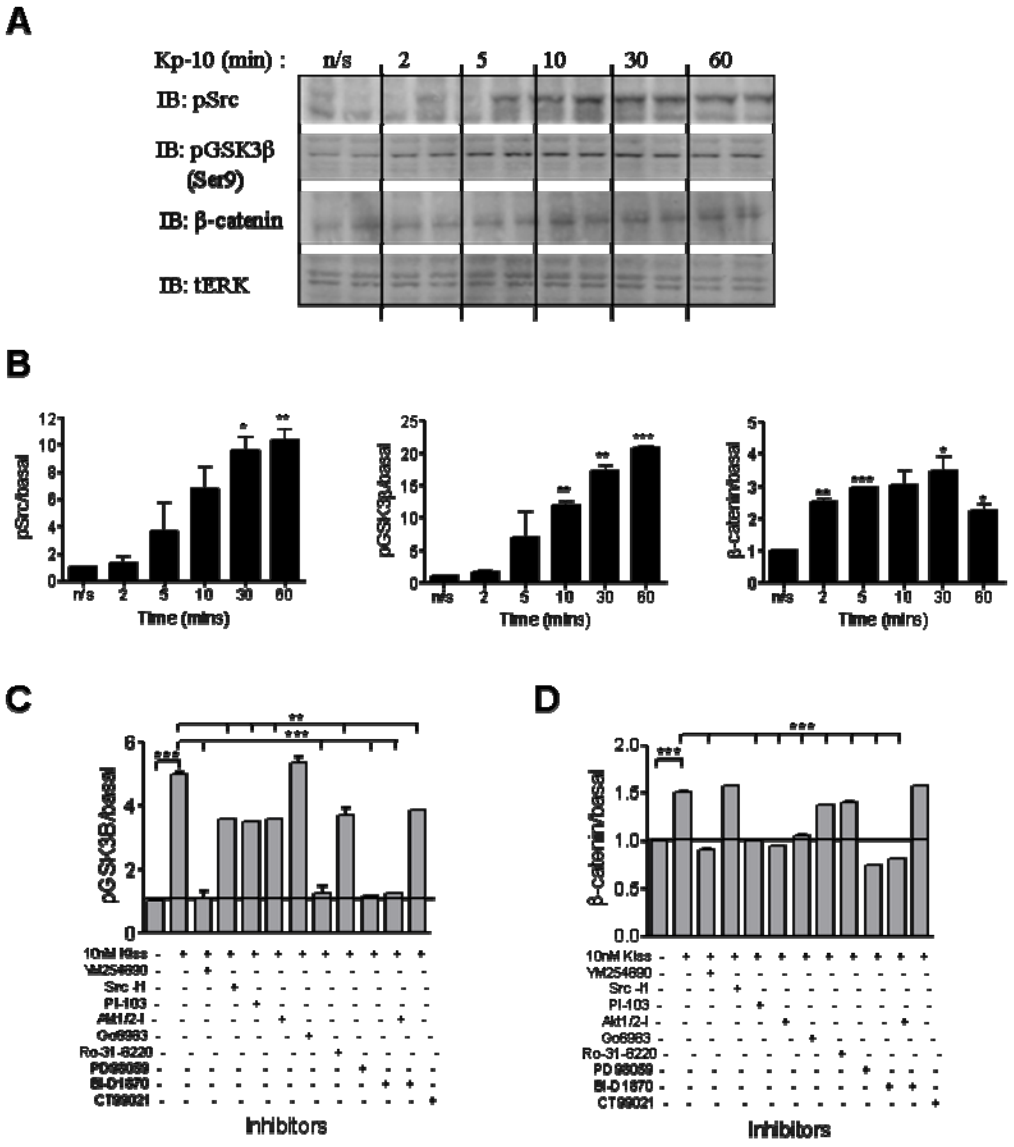

Figure 65. Kisspeptin modulates GSK3β and β-catenin pathways. (A) Kisspeptin phosphorylates Src (n=3) and GSK3β (Ser^9; n=4), while increasing β-catenin protein within the cytoplasm (n=4). Total ERK1/2 was used as a loading control. (B) Quantifications of western blots showing significant phosphorylation of Src at 30-60 minutes and GSK3β at 10-60 minutes. Beta-catenin is significantly increased throughout the stimulation. (C) Phosphorylation of GSK3β is blocked by 100nM YM254890 ($G_{q/11}$), 1μM Ro-31-8220 (PKC α, β, γ, ε) and 1μM BI-D1870 (p90rsk) inhibitors and decreased by 1μM Src Inhibitor 1 (Src), 1μM PI-103 (PI(3)K), 1μM Akt Inhibitor 1/2 (Akt), 20μM PD98059 (MEK) and 1μM CT99021 (GSK3β) inhibitors (n=3). (D) The increase of β-catenin is blocked by 100nM YM254890 ($G_{q/11}$), 1μM PI-103 (PI(3)K), 1μM Akt Inhibitor 1/2 (Akt/PKB), 1μM Go6983 (PKC α, β, γ, δ, μ, ζ) and 1μM BI-D1870 (p90rsk) inhibitors and decreased by 1μM Ro-31-8220 (PKC α, β, γ, ε) and 20μM PD98059 (MEK) inhibitors (n=4).

4.2.3. Kisspeptin-10 does not activate Rho GTPases in CHO cells stably expressing human gpr-54

Since stimulation with kp-10 induced cell rounding and formation of stress fibres in this the CHO cell line, the pathways usually responsible for these activities were investigated next. Stress fibre formation is controlled via Rho GTPase family members, RhoA and Rac-1/Cdc42. RhoA causes the inactivation of MLC phosphatase by ROK phosphorylation to promote actin-myosin assembly and stress fibre formation. Rac-1/Cdc42 is known to stabilise adherens junctions via inhibition of IQGAP1, which binds β-catenin and cadherins to disrupt binding to the actin cytoskeleton (Kaibuchi et al., 1999; Sah et al., 2000). As kisspeptin has been shown to regulate β-catenin via a pathway that promoted adherens junction formation, this pathway may also be active. However, no significant increase of RhoA or Rac-1/Cdc42 could bee seen in these cells when stimulated with 10nM kp-10 (Fig. 66). Therefore a separate pathway must regulate stress fibre formation.

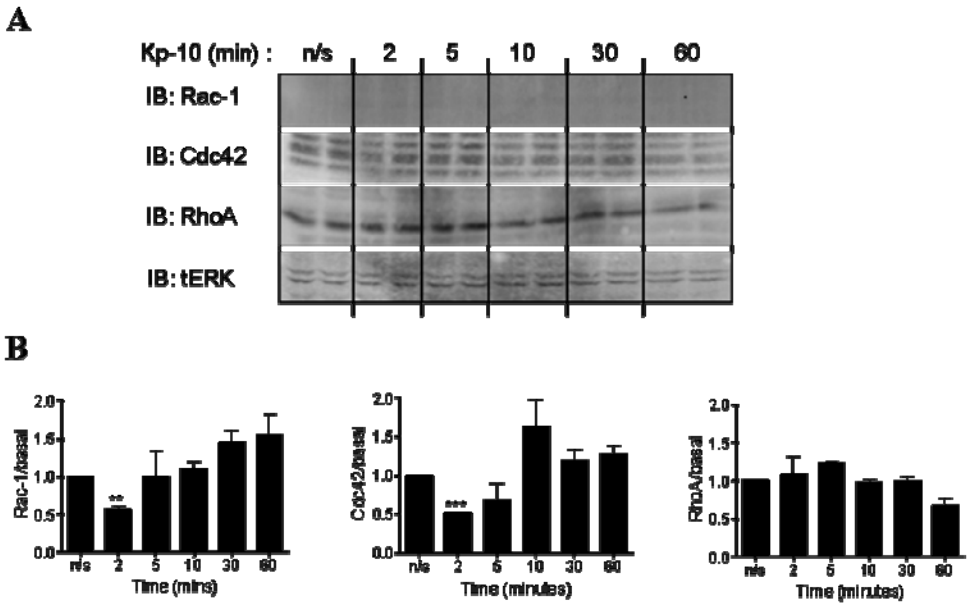

Figure 66. Kisspeptin does not activate the Rho GTPase family. (A) Kisspeptin does not activate Rac-1, Cdc42 or RhoA (n=3). Total ERK1/2 is used as a loading control. (B) Quantifications of western blots confirming no activation.

4.2.4. Kisspeptin-10 activates a more limited spectrum of pathways in GnRH neuronal cells

Pathways delineated in the CHO cells, were next tested in a more physiologically relevant cell line. The GnRH neuronal cell line, GT_{1-7} was used since gpr-54 has been shown to be present on 80% of GnRH neurons (Irwig et al., 2004). However, as little gpr-54 mRNA could be detected in these cells by RT-PCR, the mouse receptor was stably expressed to increase the output signal. To test this cell line, receptor binding was again examined, and kp-10 could bind with an IC_{50} of $4.4x10^{-8}$M. Kp-10 could also stimulate IP production and intracellular calcium release in these cells (Fig. 67). However, due to a relatively low amount of total binding suggesting low receptor number compared to the CHO cells; 100nM kp-10 was used for protein studies in these cells. The first proteins tested were from the MAPK pathway, where a significant phosphorylation of ERK1/2 was seen upon stimulation with kp-10, with a maximal response at 5 minutes. The use of inhibitors revealed that this involved PKC, Src, EGFR transactivation and PI(3)K. A significant phosphorylation of NFκB and GSK3β (Ser^9) was also induced, with maximal responses at 30 and 10 minutes, respectively (Fig. 68). NFκB phosphorylation was shown by inhibitors to be $G_{q/11}$ mediated but did not involve PKC, MEK, EGFR or Src whereas GSK3β phosphorylation was via a similar pathway to ERK1/2 suggesting p90rsk may be involved as above. However, no significant activation of Src, Akt or β-catenin could be found in these cells (Fig. 68). This is in accordance with the observation that kp-10 does not influence migration within these cells, and therefore the pathway stimulating calcium influx may be preferentially activated in neuronal cells. However, as secretion of GnRH could not be detected from this cell line, the effect of these signals on secretion could not be assessed. Work on other GnRH neuronal cell lines is needed to clarify the role gpr-54 signalling in the regulation of secretion.

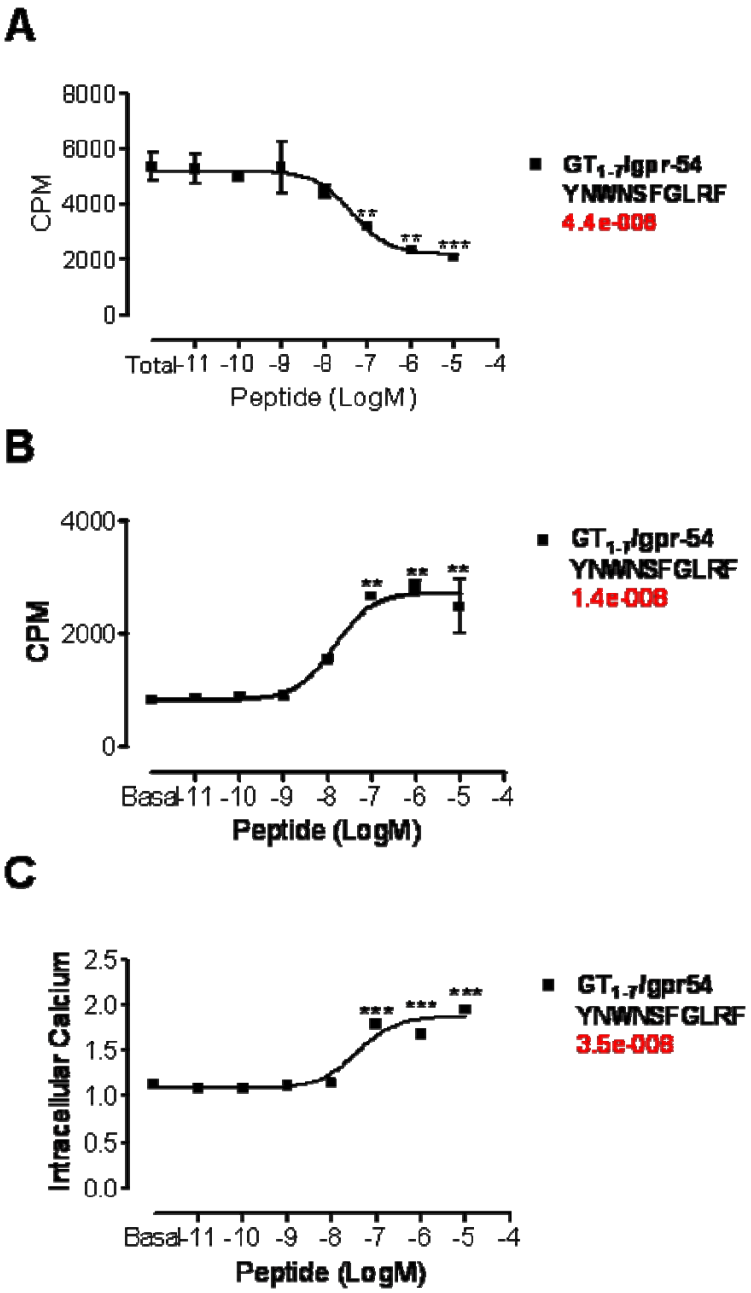

Figure 67. Kisspeptin binds to and activates gpr-54 in GT_{1-7} neuronal cells stably expressing the mouse receptor. (A) Kisspeptin binds the mouse receptor with an affinity of 44nM in these cells (n=5). (B, C) Kisspeptin can also activate the mouse receptor to stimulate IP and intracellular calcium release with an EC_{50} of 14nM and 34nM, respectively (n=5).

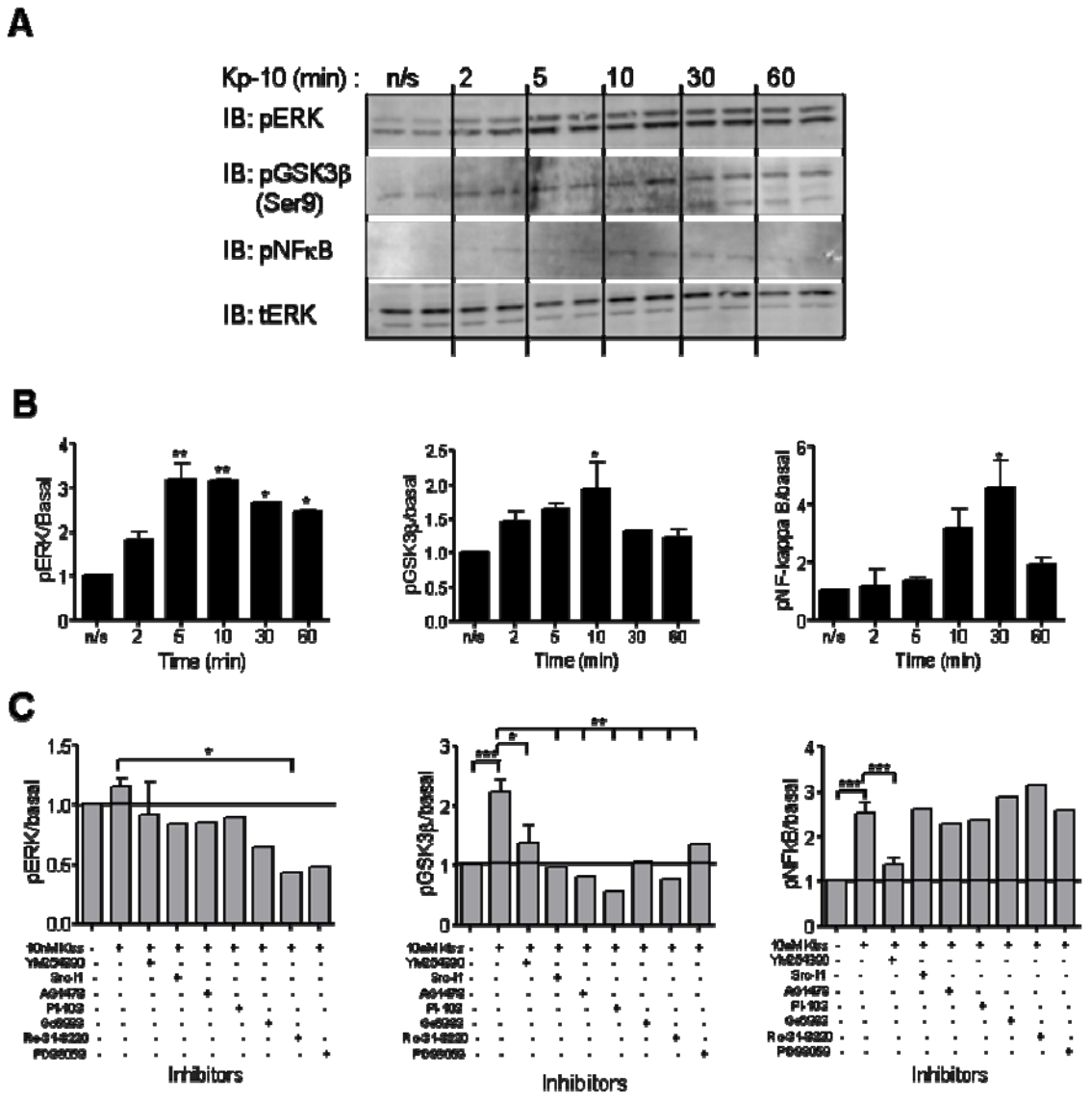

Figure 68. Kisspeptin activates a subset of signalling molecules in GnRH neuronal cells stably expressing mouse gpr-54. (A) Kisspeptin phosphorylates ERK1/2 (n=5), GSK3β (Ser^9; n=3) and NFκB (n=3) in $GT_{1\text{-}7}$/gpr-54 cells. Total ERK1/2 was used as a loading control. (B) Quantification of western blots showing ERK1/2 phosphorylation from 5-60 minutes, GSK3β phosphorylation at 10 minutes and NFκB phosphorylation at 30 minutes. (C) ERK1/2 (n=5) and GSK3β (n=3) phosphorylation was decreased by 100nM YM254890 ($G_{q/11}$), 1μM Src Inhibitor 1 (Src), 1μM AG1478 (EGFR), 1μM PI-103 (PI(3)K), 1μM Go6983 (PKC α, β, γ, δ, μ, ζ), 1μM Ro-31-8220 (PKC α, β, γ, ε) and 20μM PD98059 (MEK) inhibitors. This was only significant for GSK3β. Whereas NFκB phosphorylation was only blocked by 100nM YM254890 (Gq/11) inhibitor (n=5).

4.2.5. Kisspeptin-10 signals via migratory pathways in breast cancer and trophoblast cell lines

Since only a select number of proteins were activated in the GnRH neuronal cells, it was decided to see if the other pathways found within the model CHO cell line were active in cells from tissues were kisspeptin has peripheral actions on migration. Therefore examination of the intracellular signalling pathways in four cancer cell lines was undertaken.

B35 neuroblastoma cells

Firstly, rat neuroblastoma cells (B35) were tested to look at other cells types within the neuronal network of the brain. B35 cells are neuronal cancer cells and gpr-54 has been shown to be present within other areas of the brain. However, no stimulation of ERK1/2 could be observed upon kp-10 stimulation; however stable transfection of mouse gpr-54 DNA into these cells, did allow stimulation of IP production but still no stimulation of ERK1/2 phosphorylation was detected (Fig. 69).

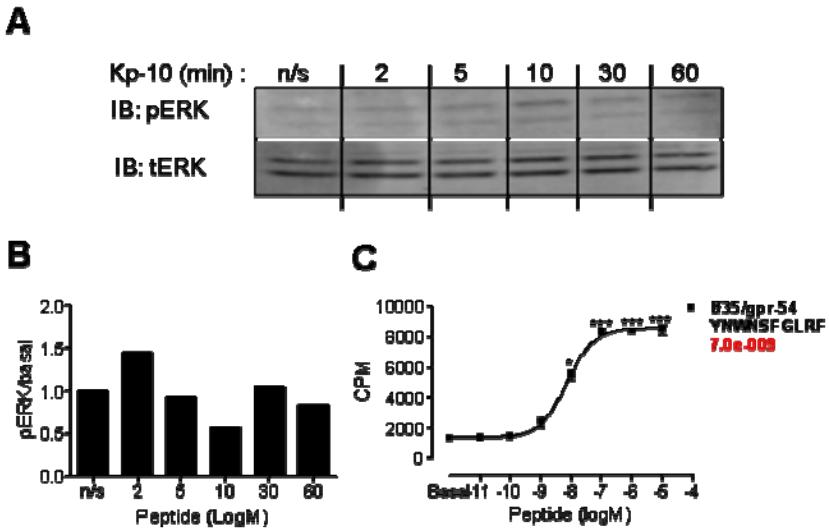

Figure 69. B35 cells do not express endogenous gpr-54. (A) 10nM Kp-10 could not stimulate ERK1/2 phosphorylation in B35 rat neuroblastoma cells (n=2) Total ERK1/2 was used as a loading control. (B) Quantification of western blots showing no activation. (C) Kp-10 could stimulate IP release in transfected B35 cells in a dose dependant manner (n=3)

Neuroendocrine GH3 somatotropes

Next, rat pituitary adenoma somatotrope cells, GH3, were tested to assess if gpr-54 was present within the pituitary. GH3 cells are secretory somatotropic cancer cells and gpr-54 has been hypothesised to be present on this cell type; however no phosphorylation of ERK1/2, JNK or p38MAPK could be seen at any concentration suggesting there was little or no receptor in these cells (Fig. 70). Next other cell types of pituitary origin were utilised. $L\beta T_2$ and αT_3 gonadotropes were assessed by PCR to examine if kp-10 is acting directly within the pituitary to release LH and FSH but no gpr-54 mRNA could be detected (data not shown).

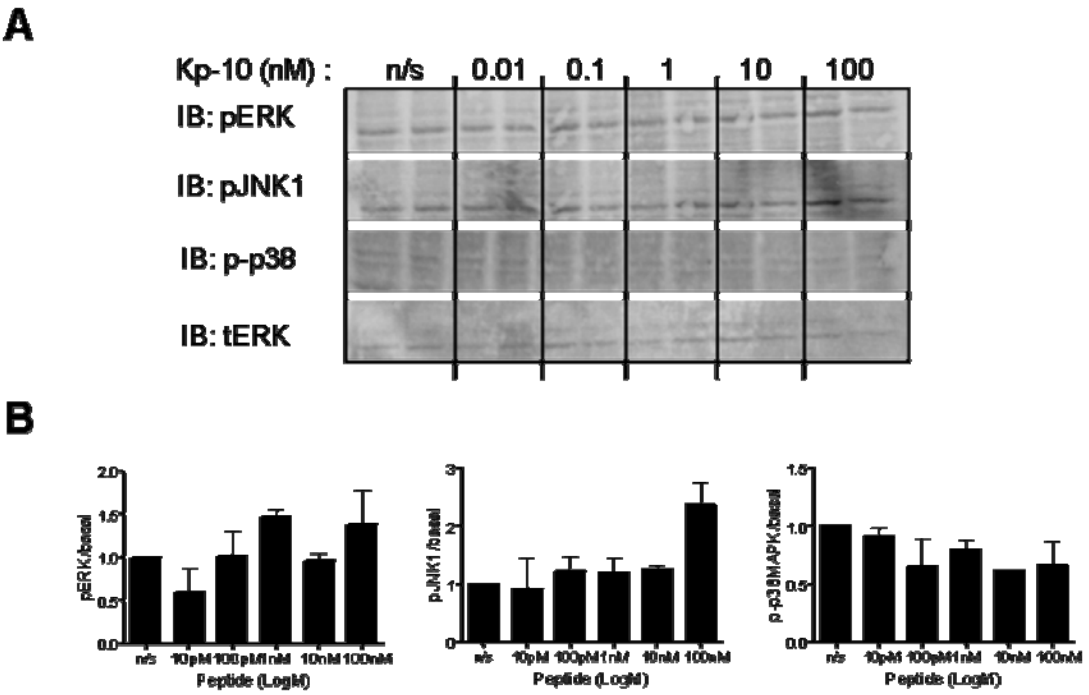

Figure 70. Gpr-54 is not present in GH3 rat pituitary adenoma cells. (A) Kp-10 does not phosphorylated ERK1/2, JNK1 or p38MAPK at any dose in GH3 cells at 10 minutes (n=2). Total ERK1/2 was used as a loading control. (B) Quantifications of western blots confirming lack of activation.

Ishikawa endometrial cancer cells

As kp-10 is known to act as an inhibitor of metastasis, a human endometrial adenocarcinoma cell line (Ishikawas) was examined but again no phosphorylation of ERK1/2 or JNK could be detected (Fig. 71).

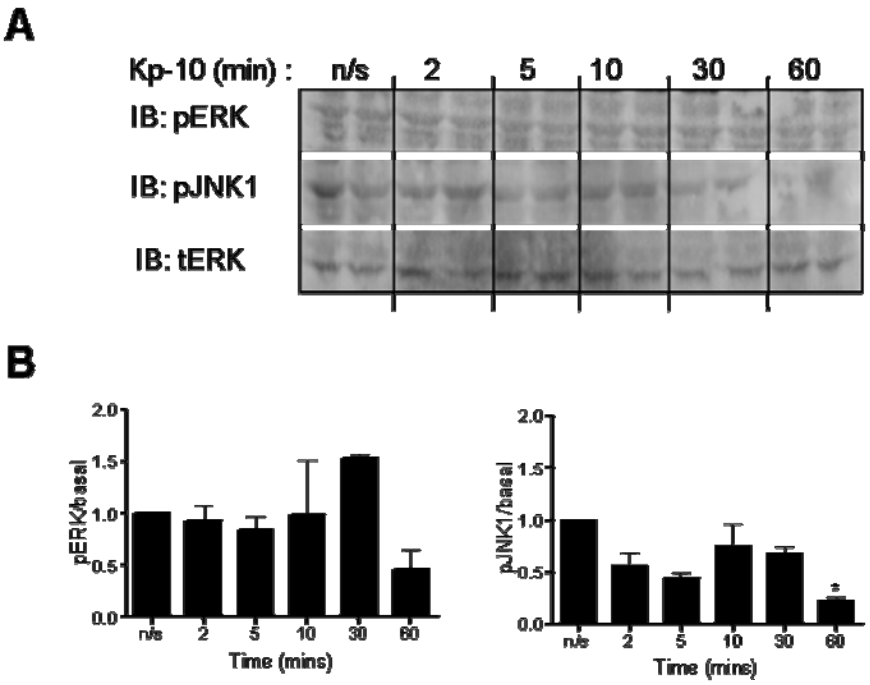

Figure 71. Gpr-54 is not present in the Ishikawa endometrial epithelial cancer cell line. (A) 10nM Kp-10 could not stimulate phosphorylation of ERK1/2 or JNK1 in Ishikawa human endometrial adenocarcinoma cells (n=2). Total ERK1/2 was used as a loading control. (B) Quantification of western blots confirming no phosphorylation.

MCF-7 breast cancer cells

Finally, to further test if kp-10 was present in cancer cell lines and to examine the pathways activated, the human breast adenocarcinoma cell line, MCF-7 was tested and kisspeptin stimulation induced phosphorylation of ERK1/2 with a similar fold increase to CHO/gpr-54 cells. This phosphorylation was both dose and time dependant. Kisspeptin could also stimulate IP production in these cells with a similar EC_{50} to CHO

cells but a less pronounced stimulation (Fig. 72). Since MCF-7 cells appeared to possess an active receptor for kp-10, other pathways activated in the model CHO cell line were analysed. Kp-10 could also significantly induce phosphorylation of FAK, GSK3β and stimulated β-catenin release. 10nM and 100nM doses of kp-10 stimulated phosphorylation of FAK at 10 minutes and 60 minutes, respectively. Phosphorylation of GSK3β was stimulated by 10nM kp-10 at 10 minutes and by 100nM kp-10 at the later time point of 60 minutes. Finally, β-catenin release was induced with 100nM kp-10 from 30-60 minutes and only marginally stimulated by 10nM kp-10 at 10 minutes (Fig. 73). Therefore, MCF-7 cells express gpr-54 and can regulate some of the migratory pathways seen to be activated by kisspeptin in the model cell line previously examined.

Choriocarcinoma cell lines: JEG, JAR and BeWo cells

As well as looking at these pathways in cancer cell lines; placental trophoblast cell lines were also utilised as kisspeptin has been shown to inhibit trophoblast invasion within the placenta. Therefore, four placental cell lines including three choriocarcinoma derived cell lines and one immortalised first trimester trophoblast cell line were examined. Firstly, the three choriocarcinoma cell lines were analysed as they are both cancerous and placental in origin. JEG-3 cells were found to have no stimulation of IP production and to induce no phosphorylation of ERK (Fig. 74), suggesting the receptor may not be expressed in this cell line. The other two choriocarcinoma cells lines, BeWo and JAR cells were also found to be incapable of phosphorylating ERK1/2 in response to kp-10 stimulation (Fig. 74).

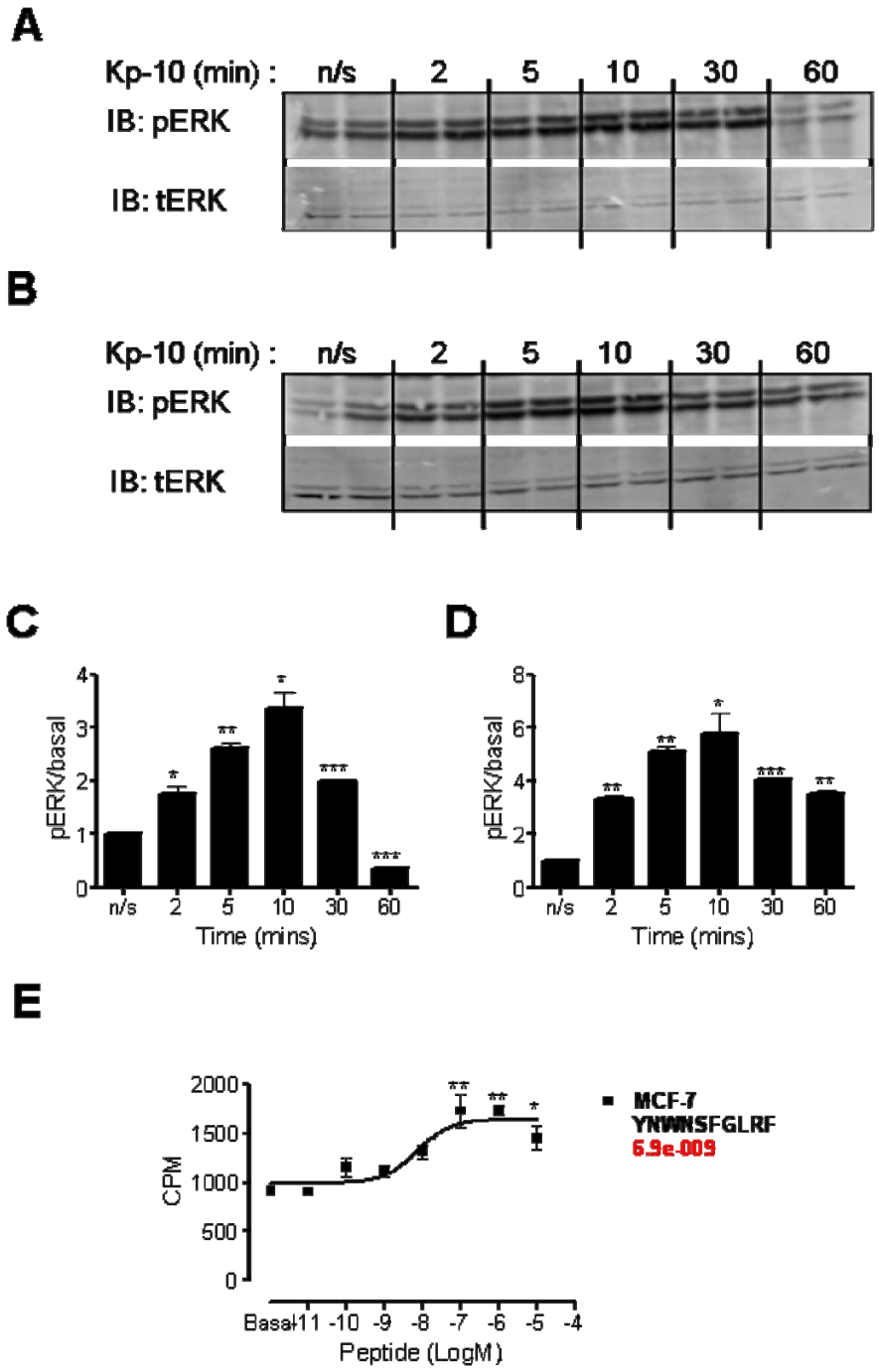

Figure 72. Endogenous gpr-54 is present in MCF-7 breast cancer cells. (A) 10nM Kp-10 phosphorylates ERK1/2 from 2-30 minutes peaking at 10 minutes (n=4). (B) 100nM Kp-10 phosphorylates ERK1/2 from 2-60 minutes peaking after 10 minutes (n=4). Total ERK1/2 was used as a loading control. (C, D) Quantifications of western blots for 10 and 100nM confirming activation of ERK1/2. (E) Kp-10 also stimulates IP release in a dose dependant manner with an EC_{50} of 6.9nM (n=3).

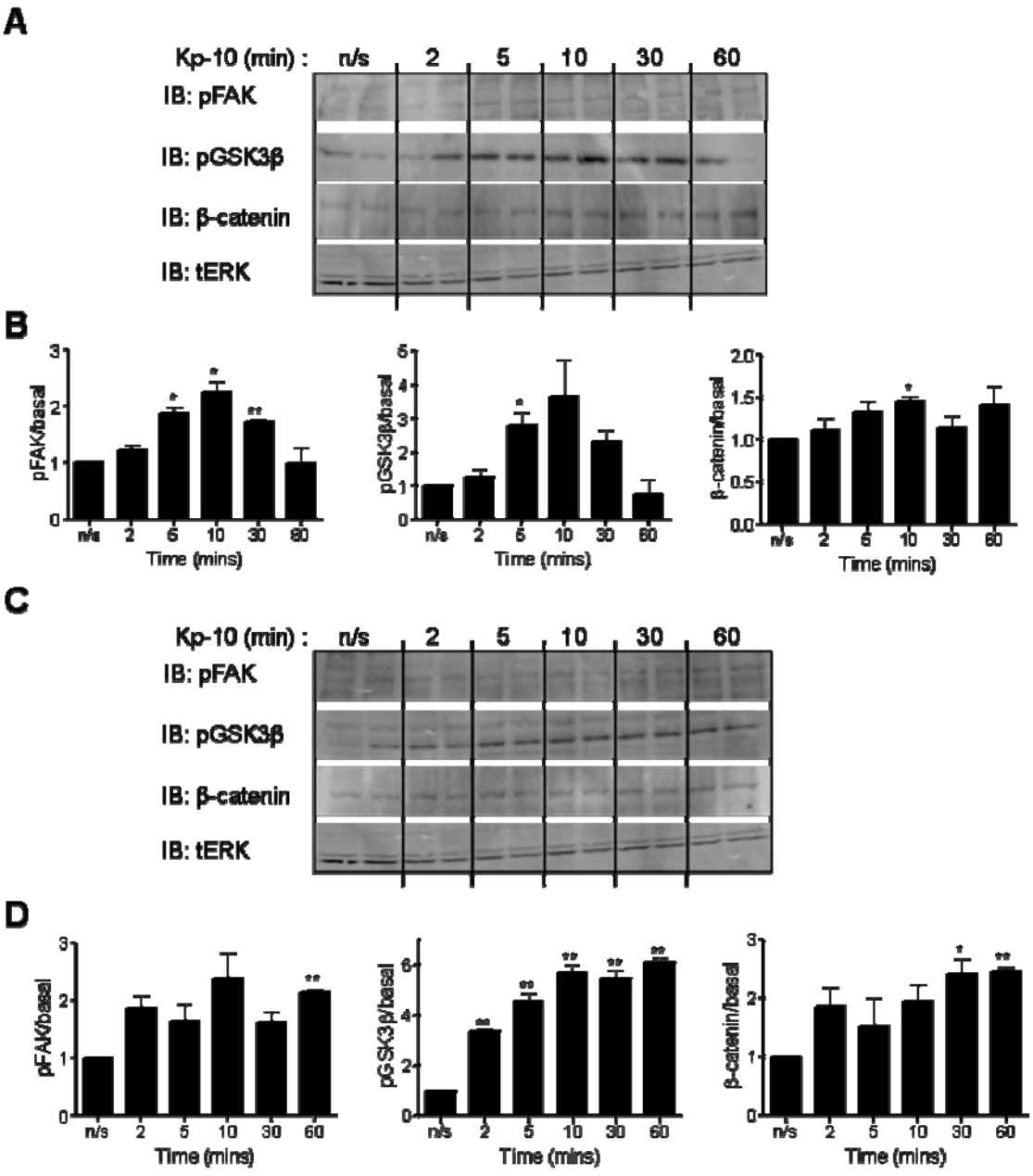

Figure 73. Kp-10 activates migratory signalling proteins in MCF-7 cells. (A) 10nM kp-10 phosphorylates FAK (n=3), GSK3β (n=4) and increases β-catenin (n=3) in the cytoplasm. Total ERK1/2 was used as a loading control. (B) Quantifications of western blots showing each increase peaks at 10 minutes. (C) 100nM kp-10 phosphorylates FAK (n=3), GSK3β (n=4) and increases β-catenin (n=3) in the cytoplasm to a greater extent than 10nM. (D) Quantifications of 100nM kp-10 western blots showing prolonged activation throughout the 60 minute stimulation.

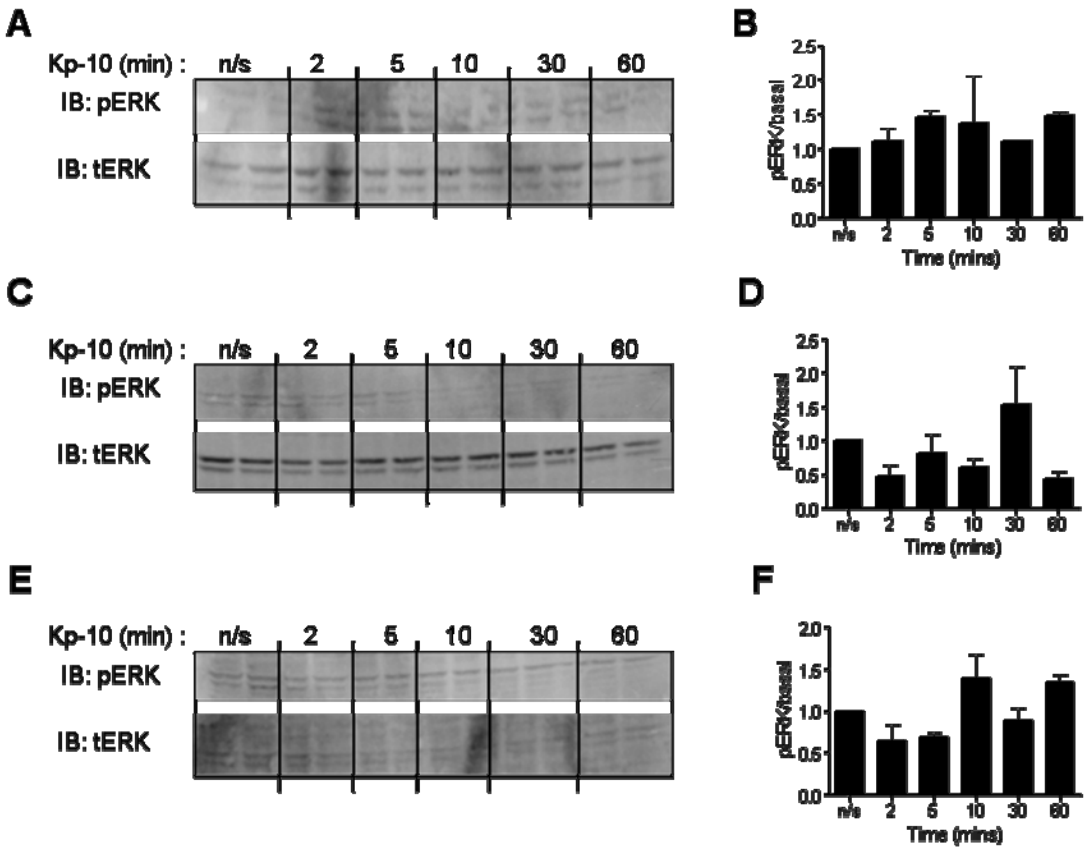

Figure 74. Gpr-54 expression is low in choriocarcinoma cell lines. (A, B) 100nM kp-10 cannot stimulate ERK1/2 in JEG-3 choriocarcinoma cells (n=2). (C, D) 100nM kp-10 is unable to phosphorylate ERK1/2 in BeWo choriocarcinoma cells (n=4). (E, F) 100nM kp-10 could not phosphorylate ERK1/2 in JAR choriocarcinoma cells (n=2). Total ERK1/2 was used as a loading control.

HTR8SVneo immortalised human first trimester extravillious trophoblast cells

As it appeared that the cancerous phenotype of these cells lines was deleterious to gpr-54 expression, research was focused on the immortalised human first trimester trophoblast cell line termed HTR8SVneo. In these cells, kp-10 was shown to bind the receptor with a 10-fold lower affinity than in the CHO cells of 1.27×10^{-7}M, but curiously could not produce an IP response. Kp-10 could however stimulate intracellular calcium release with an EC_{50} of 4.3×10^{-7}M, which is consistent with the binding affinity (Fig. 75). This may be via ion channel influx of calcium as seen in GnRH neurons.

Next the downstream signalling pathways were examined to see if the migratory proteins were activated. For the MAPK pathway, 100nM kp-10 could stimulate phosphorylation of ERK1/2 and p38MAPK. ERK1/2 was phosphorylated at 10 minutes and p38MAPK was more robustly phosphorylated at 10 minutes. Using inhibitors to delineate the pathway for these phosphorylations, $G_{q/11}$, Src, EGFR, PI(3)K, PKC and MEK inhibitors were shown to block ERK1/2 phosphorylation and $G_{q/11}$, EGFR, PI(3)K and MEK blocked p38MAPK phosphorylation (Fig. 76). This pathway is similar to that observed in the CHO cells as EGF receptor transactivation was also needed for the activation of MAPK proteins in those cells. Next the migratory proteins were examined; kp-10 could induce phosphorylation of NFκB, FAK, Src, and GSK3β as well as increase β-catenin in the cytoplasm. NFκB phosphorylation was shown to be stimulated by kp-10, peaking at 10 minutes. The pathway for this phosphorylation was similar to the CHO cells as $G_{q/11}$ and PKCε inhibitors ablated this phosphorylation. However, in these cells this phosphorylation was also inhibited by Src, EGFR, PI(3)K and ERK showing again the importance of EGFR transactivation and the MAPK pathway in the trophoblast cell (Fig. 77). FAK phosphorylation was maximally increased at 10 minutes and also appeared dependant on $G_{q/11}$, EGFR and ERK with input from PKC (α, β or γ; Fig 77). Src was next shown to be phosphorylated upon stimulation with kp-10 with a maximal increase at 10 minutes, which was sustained until 60 minutes (Fig. 78). However, the most important mechanism within these cells appears to be the phosphorylation of Ser^9 within GSK3β, which was increased significantly at 5 minutes. This phosphorylation was inhibited by a multitude of factors including $G_{q/11}$, Src, EGFR, PI(3)K, PKC and MEK (Fig. 78). This suggests that PKC, Akt and p90rsk are activated by kp-10 within these cells to phosphorylate Ser^9. Although, none of these proteins appear to regulate β-catenin as only the $G_{q/11}$ inhibitor blocks the kp-10 induced increase at 30-60 minutes within the cytoplasm (Fig. 78). Therefore, GSK3β inhibition may be activating a different mechanism in the HTR8SVneo cells than is activated in the CHO cell line where β-catenin is regulated. However, further investigation is needed to elucidate this mechanism fully.

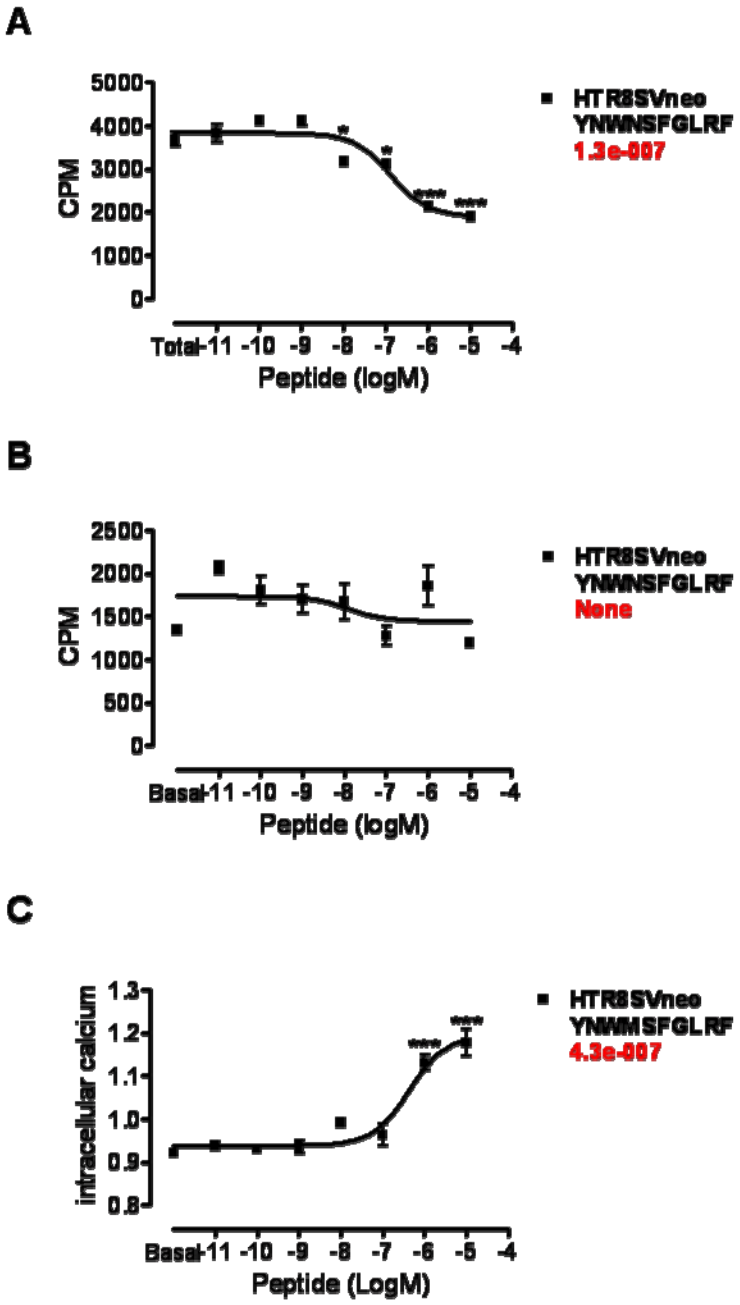

Figure 75. Endogenous gpr-54 expressed within HTR8SVneo immortalised human trophoblast cells. (A) Kp-10 can bind gpr-54 with an affinity of 127nM in first trimester trophoblasts (n=6). (B) Kp-10 does not stimulate IP release in these cells (n=3) but (C) does induce intracellular calcium release with an EC_{50} of 433nM (n=3).

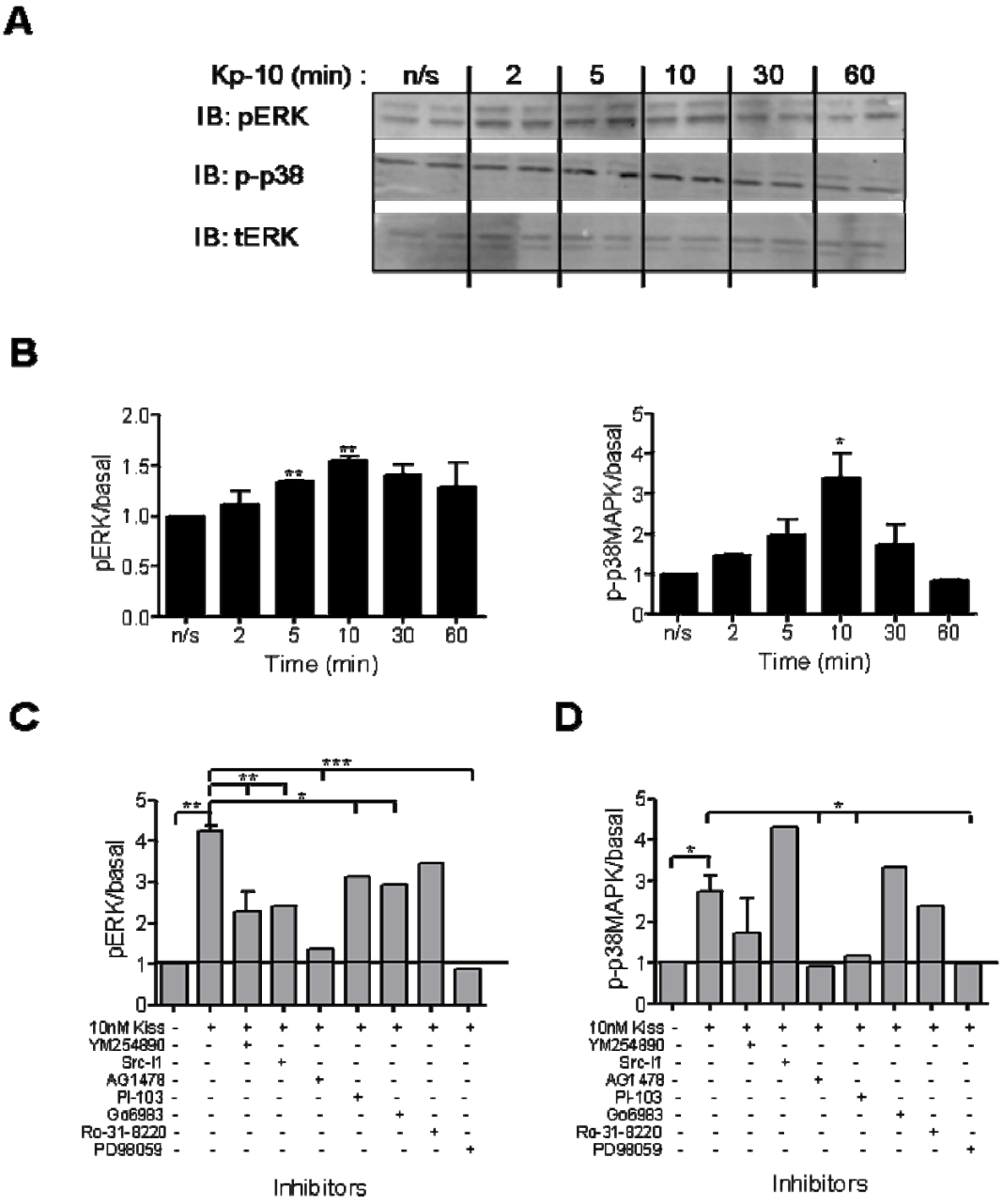

Figure 76. Kp-10 activates MAPK pathways in HTR8SVneo human trophoblast cells. (A) Kp-10 can phosphorylate ERK1/2 (n=4) and p38MAPK (n=3) in HTR8SVneo cells. Total ERK1/2 was used as a loading control. (B) Quantifications of western blots showing increase over the 60 minute stimulation peaking between 5-10 minutes. (C) Phosphorylation of ERK1/2 was significantly blocked by 1μM AG1478 (EGFR) and 20μM PD98059 (MEK), and 100nM YM254890 ($G_{q/11}$), 1μM Src Inhibitor 1(Src), 1μM PI-103 (PI(3)K) and 1μM Go6983 (PKC α, β, γ, δ, μ, ζ) caused a significant decrease (n=3). (D) P38MAPK phosphorylation was blocked by 1μM AG1478 (EGFR), 1μM PI-103 (PI(3)K) and 1μM PD98059 (MEK) inhibitors (n=3).

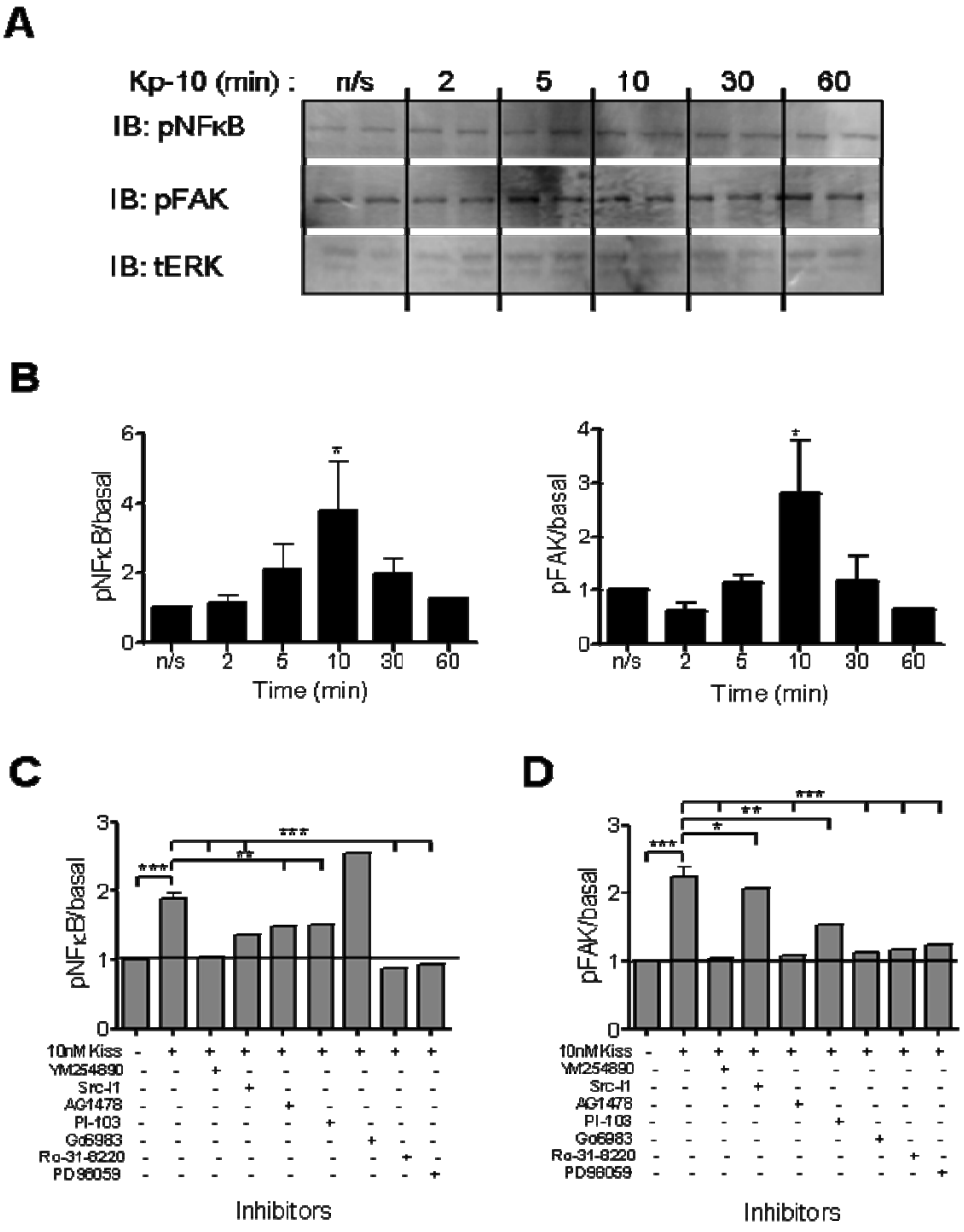

Figure 77. Kp-10 phosphorylates NFκB and FAK migratory proteins in HTR8SVneo human trophoblasts. (A) Kp-10 phosphorylates NFκB and FAK at 10 minutes (n=3). Total ERK1/2 was used as a loading control. (B) Quantifications of western blots showing a significant increase at 10 minutes. (C) Phosphorylation of NFκB is blocked by 100nM YM254890 ($G_{q/11}$), 1μM Src Inhibitor 1 (Src), 1μM AG1478 (EGFR), 1μM PI-103 (PI(3)K), 1μM Ro-31-8220 (PKC α, β, γ, ε) and 20μM PD98059 (MEK) inhibitors (n=3). (D) FAK phosphorylation is ablated by 100nM YM254890 ($G_{q/11}$), 1μM AG1478 (EGFR), 1μM Go6983 (PKC α, β, γ, δ, μ, ζ), 1μM Ro-31-8220 (PKC α, β, γ, ε) and 20μM PD98059 (MEK) and slightly decreased by 1μM PI-103 (PI(3)K) inhibitors (n=3).

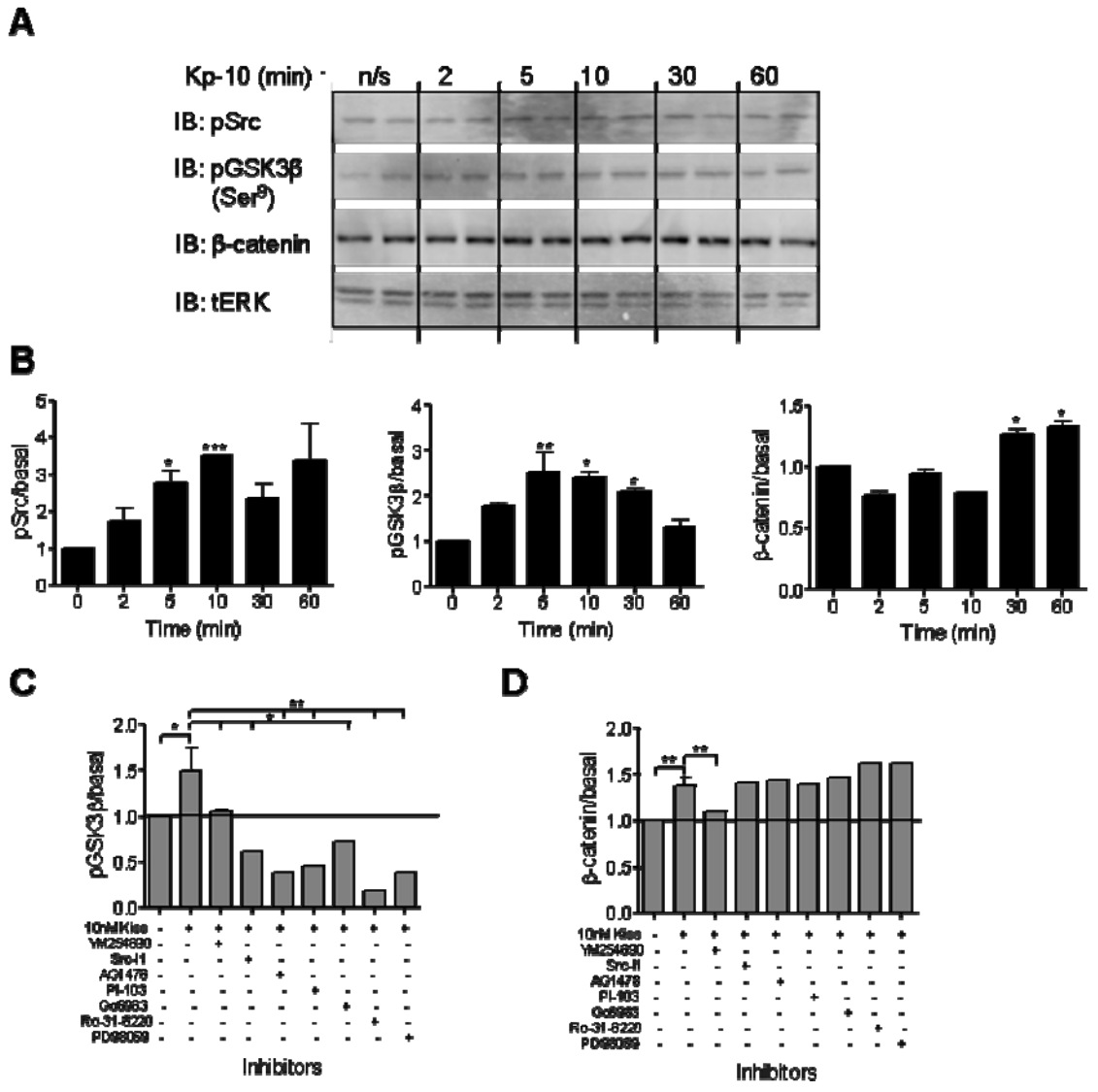

Figure 78. Kp-10 mediates GSK3β and β-catenin pathways in HTR8SVneo human trophoblast cells. (A) Kp-10 phosphorylates Src and GSK3β at 5-30 minutes and increases β-catenin in the cytoplasm after 30 minutes (n=4). Total ERK1/2 is used as a loading control. (B) Quantifications of western blots showing significantly increased phosphorylation and protein. (C) Phosphorylation of GSK3β is ablated by 100nM YM254890 (Gq/11), 1μM Src Inhibitor 1 (Src), 1μM AG1478 (EGFR), 1μM PI-103 (PI(3)K), 1μM Go-6983 (PKC α, β, γ, δ, μ, ζ), 1μM Ro-31-8220 (PKC α, β, γ, ε) and 20μM PD98059 (MEK) inhibitors (n=3). (D) β-catenin protein within the cytoplasm is decrease by the 100nM YM254890 ($G_{q/11}$) inhibitor only in trophoblast cells (n=3).

4.3. Discussion

Kisspeptin is known to be important for the secretion of GnRH in the hypothalamus (Gottsch et al., 2004; Irwig et al., 2004; Messager et al., 2005) and has also been shown to affect the migration of a variety of cells including CHO cells, trophoblasts and cancer cell lines (Bilban et al., 2004; Hiden et al., 2007; Hori et al., 2001; Kotani et al., 2001; Lee et al., 1996). Until now the intracellular proteins and pathways for these processes have not been fully investigated due to large drive to examine the physiological effects of kisspeptins and gpr-54 in a variety of animal models. However, understanding the signalling mechanisms activated by gpr-54 in cells from different tissues where kp-10 is known to act may help to clarify these physiological effects and differential actions associated with gpr-54 activation.

In order to address this issue, the intracellular proteins activated by kp-10 were investigated in CHO cells and GT_{1-7} neuronal cells stably expressing gpr-54, and a variety of cancer and trophoblast cell lines. The majority of the above pathway analysis has been undertaken using western blot and inhibitor studies as these are commonly used methods; however alternative techniques could have been used to obtain the findings within this chapter. Other methods to inhibit the proteins within the pathways studied may have been utilised, such as introduction of dominant negative mutants or short-interfering RNA (siRNA). Dominant negative proteins are proteins with inactivating mutations (negatives), which when over-expressed in cells can cause inhibition of the wild-type protein function (dominant). Dominant negatives are useful as they can be more specific than inhibitors, even enabling separate analysis of different isoforms of proteins. An alternative method would be to use siRNAs, which when transfected into cells can bind to the RNA-induced silencing complex (RISC), which then cleaves the target mRNA complementary to the bound siRNA, effectively silencing the gene of interest. However, the knockdown of the gene expression is not always complete, which may lead to less effective results than with inhibitors; on the other hand they can give a more specific inhibition.

Another area where alternative techniques may also have been employed is within the detection of the protein levels within samples. Quantitative proteomic analysis may have been used to obtain information on all the proteins within a sample. Mass spectrometry-based proteomics is used for this purpose, with the most common form being stable isotope labeling with amino acids in cell culture (SILAC). This involves the culture of two cell populations were one is given normal amino acids in growth medium and the other given heavy isotope amino acids (to increase the mass). Once combined, chemically identical proteins with different masses can be identified by mass spectrometry and the ratio used to calculate the abundance of a protein in a sample. This technique can also be used to examine the overall phosphorylation status of a sample. Alternatively, 2-D gel electrophoresis could have been used to separate proteins by charge via isoelectric focusing and then by mass via SDS-PAGE. This allows all the proteins within a sample to be quantified at once. Also, if protein samples are labeled with different flourophores they can be run on the same gel, decreasing the amount of inter-gel differences and allowing variations between samples to be calculated.

However, as mentioned above western blots and inhibitor were utilised and it was found that the main signalling pathways activated in all cell types are MAPK, GSK3β and NFκB signalling with some influence of FAK signalling in certain cell types. The mechanism of this activation appears consistent between tissues with most cell lines exhibiting EGF receptor transactivation. This suggests that gpr-54 signalling, although cell type dependant, recruits similar pathways to facilitate a range of physiological outcomes.

The main pathway activated in all cell types that responded to kp-10 was the MAPK signalling cascade. In the CHO/gpr-54 cell line, representative members of the MAPK family were activated, with a very robust stimulation of ERK1/2. ERK1/2 activation involved a mechanism where EGFR transctivation is centrally important (Fig. 79). However, in MCF-7 breast cancer cells and HTR8SVneo trophoblast cells only activation of ERK1/2 and p38MAPK was induced by kp-10 with no stimulation of the

JNK pathway (Fig. 80). In GT_{1-7} neuronal cells, of the MAPKs, only ERK1/2 activation was detectable and this also involved EGF receptor transactivation (Fig. 81). Transactivation has previously been shown in these cells in response to GnRH where p90rsk was also up regulated and may explain why GSK3β is inhibited in this cell line (Shah et al., 2003). Also, in GT_{1-7} cells there appears to be a constitutive phosphorylation of ERK1/2, due to the fact that inhibitors used in these cells reduced this phosphorylation below basal levels. In these cells it is known that diverse stimuli such as lead can induce ERK phosphorylation as can prion protein clustering (Monnet et al., 2004; Zhang et al., 2003), also Ark, a receptor tyrosine kinase has been shown to activate ERK1/2 to regulate anti-apoptotic effects in these cells (Allen et al., 2002; Allen et al., 1999).

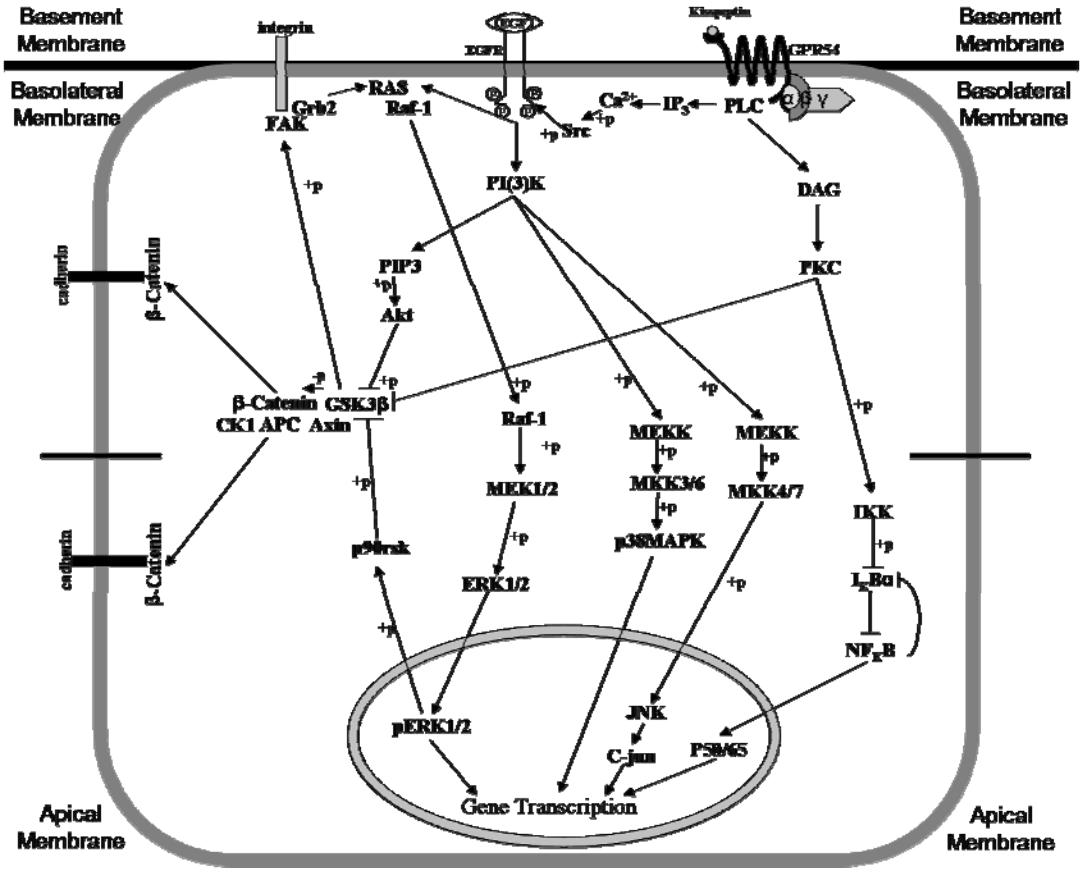

Figure 79. Schematic diagram of signalling pathways activated by kp-10 in CHO cells stably expressing the human gpr-54. Kp-10 mediates MAPK, GSK3β, NFκB and FAK.

Activation of MAPK signalling by kp-10 within all cell types studied was further shown to be pivotal to the inhibition of GSK3β via Ser^{9} phosphorylation to release β-catenin in to the cytoplasm in CHO and trophoblast cells (Fig. 79 and 80). This occurs via ERK1/2 phosphorylating p90rsk at Thr^{573} to activate an auto-phosphorylation event to fully activate p90rsk and at the same time ERK1/2 also primes GSK3β for Ser^{9} phosphorylation by p90rsk (Anjum and Blenis, 2008). This then becomes a pseudo-substrate for GSK3β that can occupy the anoxyion binding pocket, but cannot be further phosphorylated as a proline is situated at the first GSK3β phosphorylation site four residues N-terminally to Ser^{9} in place of a Thr/Ser residue. This can then compete with β-catenin to inhibit GSK3β (Frame and Cohen, 2001; Pearl and Barford, 2002). MAPK may also influence GSK3β inhibition via p38MAPK which can phosphorylate a specific Ser^{389} residue to cause the release of β-catenin into the cytoplasm (Thornton et al., 2008); however this would need to be tested in all the cell types.

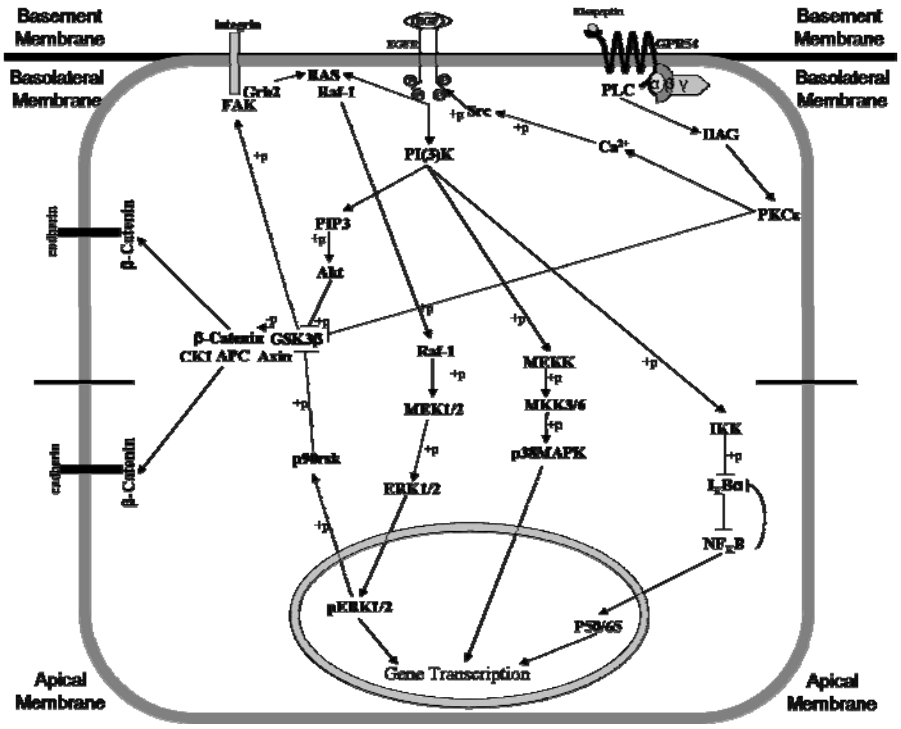

Figure 80. Schematic diagram of multiple pathways activated by kp-10 in trophoblast cells. Kp-10 mediates MAPK, GSK3β, NFκB and FAK signalling in HTR8SVneo cells. Note the absence of inositol phosphate production and JNK activation and a preference for PKCε isoform.

Inhibition of GSK3β and release of β-catenin into the cytoplasm appears critical to kp-10 signalling in the CHO and trophoblast cell lines, with some effect of GSK3β in the GnRH neuronal cell line; however, this does not appear to be the regulation of β-catenin. In contrast, the role of GSK3β in the GnRH neuron is unclear but may involve gene transcription or regulation of the insulin pathway (Fig. 81). Whereas in the CHO and trophoblast cell lines; inhibition of GSK3β by Ser^9 phosphorylation may play an influential role on release of β-catenin into the cytoplasm. Again, similarities between cells are evident with CHO, GT_{1-7} and trophoblast cells using transactivation of the EGF receptor. In the CHO cells as well as the MAPK/p90rsk pathway, inhibition of the PI(3)K/Akt pathway members and PKC also reduces the phosphorylation of Ser^9 and blocks the release of β-catenin into the cytoplasm (Fig. 79). This suggests that multiple pathways are utilised to ensure the release of β-catenin, with p90rsk being the main contributor as blocking this pathways completely ablates this release and GSK3β phosphorylation. Whereas, blocking Akt or PKC only decreases GSK3β inhibition suggesting p90rsk can compensate for Akt/PKC but Akt/PKC cannot compensate for the loss of p90rsk in CHO cells. Due to the involvement of p90rsk this suggest that β-catenin should be assembling at adherens junctions to inhibit the movement of CHO cells (Torres et al., 1999), and this is backed up as no translocation to the nucleus could be seen in CHO cells (data not shown). Therefore, investigation of the cadherins in response to kp-10 and examining the phosphorylation status of β-catenin would resolve the mechanism utilise by β-catenin after release into the cytoplasm. Investigating the phosphorylation status would be beneficial as Akt is known to phosphorylate β-catenin to cause nuclear translocation (Fang et al., 2007).

In the trophoblast cell line, Akt and PKC also appear to be important for GSK3β phosphorylation of Ser^9 along with p90rsk, as inhibitors of Src, PI(3)K ablate this phosphorylation as does inhibitors of PKC and MEK (Fig. 81). However, none of these inhibitors affect β-catenin release suggesting that GSK3β inhibition is not important for β-catenin activation. In order to examine if this is indeed the case, inhibitors of Akt,

p90rsk and GSK3β need to be utilised in this cell line to see if they directly affect this increase in β-catenin or if GSK3β inhibition has a different role to play.

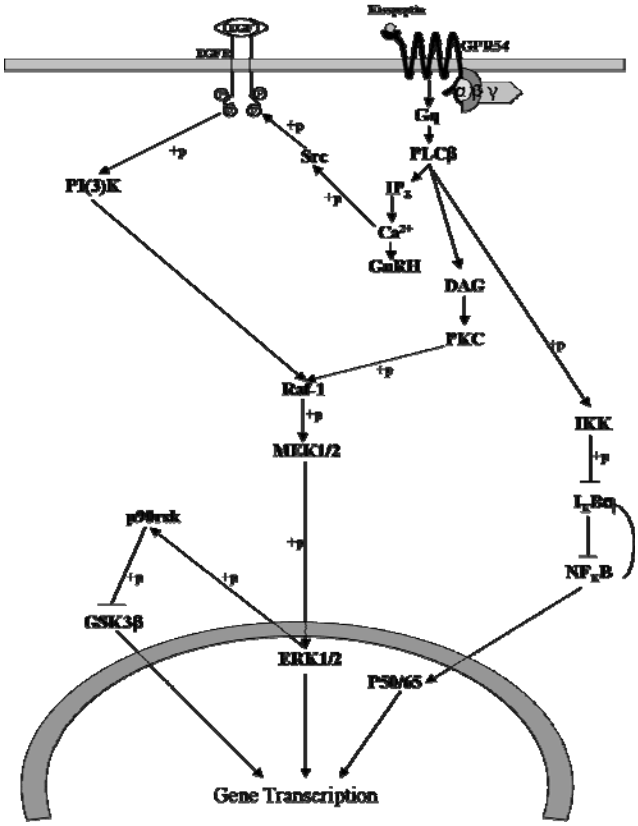

Figure 81. Schematic diagram of select pathways activated by kp-10 in GnRH neuronal cells. Kp-10 activates ERK1/2, GSK3β and NFκB in GT_{1-7} neuronal cells via PKC and EGFR.

Another characteristic of the trophoblast cell line used is apparent constitutive phosphorylation of GSK3β at Ser^9, shown as when inhibitors were used to map the pathway involved they decreased the phosphorylation status of Ser^9 to below basal levels. This may be due to activation of Akt or p90rsk via a different substrate to affect the cells migration, as the regulation of trophoblast invasion in the first trimester is probably due to a cocktail of substrates including kp-10. For example, EGF has been shown to regulate Akt and ERK1/2 in these cells and interleukins are also known to regulate extravillious trophoblasts (Paiva et al., 2009; Pollheimer and Knofler, 2005; Qiu

et al., 2004). Another reason for this constitutive phosphorylation may be GSK3β regulation of glycogen synthase within the insulin pathway, where GSK3β is known to be inhibited by Ser^{9} phosphorylation in response to the Akt/PKB pathway (Pearl and Barford, 2002).

The regulation of GSK3β via ERK1/2 and p90rsk and any other actions of the MAPK pathway appears to be important in the CHO and HTR8SVneo immortalised trophoblast cells lines. In these cell lines, FAK is also regulated by kp-10 via phosphorylation at Tyr^{925}. This phosphorylation is known to create a SH2 binding site for the Grb2-SOS complex within FAK. As SOS is a nucleotide exchange factor this can increase the activation of RAS to increase ERK1/2 phosphorylation (Fig. 82) (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996). Since this phosphorylation of FAK is dependant on ERK1/2, p90rsk, Akt and GSK3β this creates a feedback loop via FAK and Grb2 to increase ERK1/2 phosphorylation to further inhibit GSK3β and increase β-catenin within the cytoplasm (Fig. 79 and 80). It has been previously shown that GSK3β can regulate FAK phosphorylation and activation, and can even form a complex in regards to cell migration (Bianchi et al., 2005). Another role of FAK may also be to associate with integrins to form focal adhesion complexes to inhibit cell movement. FAK can be activated in response to integrin clustering via autophosphorylation of Tyr^{397}, creating a SH2 domain for binding with Src. Full activation of FAK can then occur via phosphorylation of $Tyr^{567/577}$ by Src and FAK can enhance Src activity via phosphorylation at Tyr^{418}. The FAK-Src complexes can then bind to paxillin and p130Cas at focal adhesion or regulate downstream signalling cascades such as MAPK. At focal adhesions, paxillin also binds to integrins which are bound to the ECM and vinculin which binds to the actin cytoskeleton. This complex then creates a tight connection between the ECM and cytoskeleton to modulate stresses inside and outside the cell (Parsons, 2003).

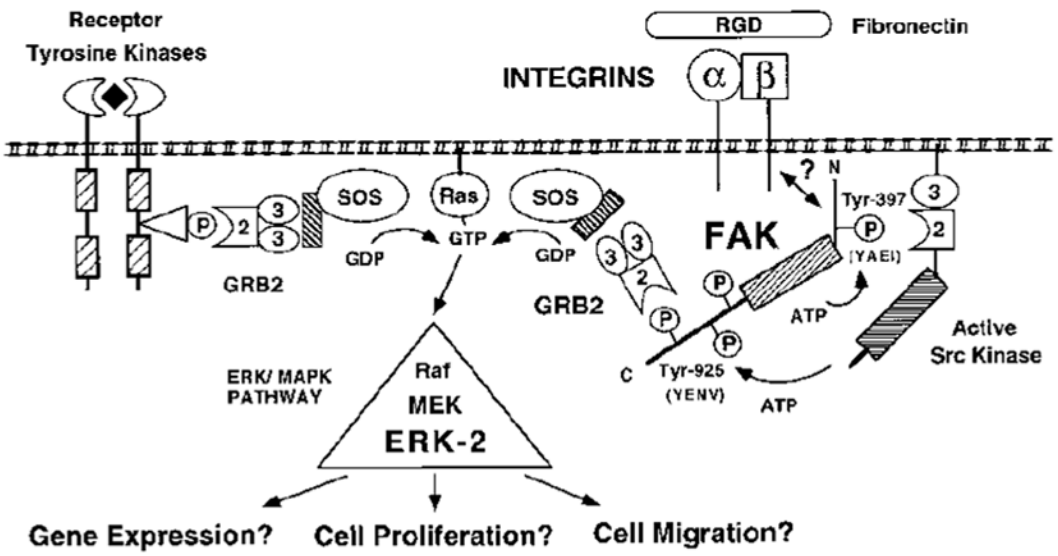

Figure 82. Diagram showing binding of Grb2 to FAK after phosphorylation of Tyr^{925}. Diagram showing phosphorylation of FAK at Tyr^{925} by Src to create a binding site for the Grb2-SOS complex. SOS then acts to exchange GTP-GDP within RAS to activate the MAPK pathways to increase phosphorylation of ERK1/2. Diagram taken from Schlaepfer, D.D and Hunter, T, *Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases* Mol Cell Biol 1996. **16**: p5623-5633.

Another pathway activated via kp-10 in all cell types tested except the MCF-7 cells, is the NFκB signalling pathway specifically dimers involving p65 as the antibody used detects Ser^{536} phosphorylation of the p65 subunit. NFκB has previously been shown in primary trophoblast cells to bind to the promoter of MMP-9 causing its activation. This then causes the breakdown of the ECM to facilitate trophoblast invasion. Kp-10 has been shown to disrupt binding of NFκB to the promoter in these cells to decrease MMP-9 expression (Yan et al., 2001). However, in the three positive cell lines tested in this thesis, there is an increase in NFκB activation suggesting that this is not the action occurring in these cell lines and that NFκB may be regulating a different set of genes. Differences can be seen in the activation of NFκB with CHO cells using only PKC mediated mechanisms and the trophoblast cells using both PKC and EGF receptor mechanisms possibly via MEK (Fig. 79 and 80). MEK has previously been shown to

activate NFκB in osteoclasts (Gingery et al., 2008). However, the mechanism in the GnRH neuron is still to be established as none of the inhibitors except for $G_{q/11}$ had an effect (Fig. 81). Also from the results obtained in this study, it appears that the most important PKC isoform is PKCε, since Ro-31-8220 is more effective than Go6983 at inhibiting the important proteins in each cell type such as NFκB, ERK1/2 and GSK3β. PKCε is a novel calcium-independent, phospholipid-dependant enzyme, known to phosphorylate Ser/Thr residues to activate downstream signalling cascades when activated by DAG. Although, most commonly known as a cardioprotectant against infarction, PKCε has also been shown to regulate calcium release and MAPK signalling (Burgos et al., 2007; Comalada et al., 2003).

In summary, kp-10 can activate a range of signalling pathways in CHO and trophoblast cells with select pathways also activated in the GnRH neuron. The main pathway activated in the CHO and trophoblast cell lines is an ERK1/2 mediated inhibition of GSK3β via p90rsk to release β-catenin into the cytoplasm. This is then further enhanced via a FAK-mediated feedback loop to increase ERK1/2 phosphorylation further. The pathways activated in these cells suggest a role for kisspeptin in the regulation of migration within these cell lines.

Chapter Five

Kisspeptin-10 inhibits migration of CHO/gpr-54, human umbilical vein endothelial cells and human placental trophoblast cells

5.0. Abstract

GnRH neurons are regulated by afferent networks of kisspeptin-producing neurons. To date, research has concentrated on signals affecting kisspeptin neurons such as sex steroids and environmental cues; however, kisspeptin was first discovered as an inhibitor of metastasis and trophoblast migration. Therefore, using an *in vitro* assay to assess cell migration; a model CHO cell line, GnRH neuronal cells along with cancer and placental cells were examined for kp-10 mediated inhibition of cell migration. The model CHO cell line and both placental cells, showed a dose-dependant inhibition of cell migration upon stimulation with kisspeptin-10. However, no effect was observed on the migration of the GnRH neuronal cells or MCF-7 breast cancer cells, suggesting differential effects of gpr-54 activation on the cytoskeleton.

In the previous chapter, kisspeptin-10 was shown to activate signalling pathways associated with migration. Inhibitors were utilised to gauge the involvement of particular proteins in this process. In both the CHO and placental cell lines, inhibition of GSK3β to release β-catenin into the cytoplasm appears critical to this mechanism. In these cells, this occurs via EGF receptor transactivation to activate the MAPK signalling cascade. Specifically, this transactivation phosphorylates ERK1/2 causing the activation of p90rsk, which can in turn inhibit GSK3β via Ser^9 phosphorylation. Inactivation of GSK3β results in release of β-catenin into the cytoplasm. Previous research has shown that p90rsk causes β-catenin to associate with cadherins at adherens junctions, suggesting a possible mechanism for kisspeptin-10 mediated inhibition of cell migration.

These results suggest that kisspeptin may activate different pathways to mediate inhibition of trophoblast cell migration which are distinct from effects on breast cancer cell migration since no migrational affects were seen in MCF-7 cells. Further confirming kisspeptins actions are cell and tissue specific.

5.1. Introduction

Kisspeptin and gpr-54 have recently emerged as potent regulators of the HPG axis via regulating the secretion of GnRH in the hypothalamus and have been shown to relay hormonal and metabolic cues to GnRH neurons. However, the *KiSS-1* gene was originally discovered as a suppressor of metastasis in a variety of cancers suggesting that the kisspeptin/gpr-54 system may regulate cellular migration and tissue invasion. Since then kisspeptin has been shown to inhibit cell migration in a model CHO cell line stably expressing the human receptor and has been shown to inhibit the migration of trophoblast cells during placentation.

The process of placentation relies on the migration of extravillious trophoblast cells into the maternal arteries of the decidua, these cells then cause remodelling of the spiral arteries to promote blood flow to the placenta and foetus during pregnancy (Lunghi et al., 2007). In order for this process to occur normally, a host of excitatory and inhibitory signals, including kisspeptin, are needed to control the degree of migration by the trophoblasts, to allow sufficient supply of blood to the foetus without damaging uterine function (Cohen and Bischof, 2007; Hiden et al., 2007). If these signals fall out of balance with each other, this can lead to either shallow migration of the trophoblast cells leading to pre-eclampsia or over-migration into the decidua, known as placental accreta (Khong, 2008; Sankaralingam et al., 2006).

It has recently been shown that kisspeptins can inhibit the migration of trophoblasts cells *in vitro* and that *KiSS-1* and *gpr-54* mRNA are present in the placental trophoblast cells (Bilban et al., 2004). *KiSS-1* and *gpr-54* mRNA have been shown to be up regulated in the first trimester of pregnancy in the placenta then down-regulated throughout the second and third trimesters (Janneau et al., 2002). This suggests a role for kisspeptin in placentation and trophoblast migration. Also in pre-eclampsia, *KiSS-1 mRNA* is increased, suggesting that kp-10 mediated inhibition of migration is overtly increased in this disease or that the target cells are inadequately responsive, contributing to the

altered migration of trophoblast cells into the maternal arteries of the decidua (Qiao et al., 2005; Zhang et al., 2006). Studies have emerged examining the cell migrational effects of kisspeptin; however these have mainly concentrated on its anti-metastatic properties.

Different mechanisms have been hypothesised for this inhibition of metastasis in several cancer cells (Table 10). Several publications have shown an increase in ERK1/2 phosphorylation and a decrease in MMP-2 expression (Masui et al., 2004; Ringel et al., 2002; Yoshioka et al., 2008). Other suggested mechanisms include antagonism of stromal cell-derived factor-1 (SDF-1) action, inhibiting the pro-migration properties of its chemokine receptor CXCR4 via inhibition of Akt phosphorylation and calcium ion mobilization. Also, the up-regulation of modulatory calcineurin-interacting protein-1 (MCIP-1), a chemokine capable of inhibiting the calcinuerin signalling pathway (Navenot et al., 2005; Stathatos et al., 2005). Another hypothesis is that kisspeptin gene expression is regulated by Specificity Protein 1 (SP1) and its co-activator DRIP130 which is located on chromosome region 6q16.3q23. When Loss of hetrozygosity (LOH) occurs at this region KiSS-1 is frequently lost from tumours and this allows metastasis to occur. This can be rescued by SP1 and DRIP130 (Mitchell et al., 2007; Shirasaki et al., 2001). Within the placenta, the regulation of MMPs is thought to be important; one study has shown that kp-10/gpr-54 activation can dephosphorylate NFκB, causing it to dissociate from the MMP-9 promoter, thereby decreasing the expression of MMP-9, and inhibiting the re-modelling of the ECM (Yan et al., 2001). However, further research is needed to delineate the signalling pathways within the placenta fully.

The results in this chapter encompass the signalling pathways identified as important for the inhibitory effects of kp-10 on cell migration using an *in vitro* assay. The results show that ERK1/2, p90rsk and GSK3β are critical to this inhibition in both a model CHO cell line and within human placental cell lines. However, this migratory pathway is not affected by kisspeptin in GnRH neuronal cells or within human MCF-7 breast cancer cells.

Cancer Type	Reference
Clear cell ovarian cancer	Prentice et al, 2007
Epithelial ovarian cancer	Hata et al, 2007
Pancreatic cancer	Masui et al, 2004
Estrogen positive breast cancer	Marot et al, 2007
Transition cell carcinoma of the bladder	Nicolle et al, 2006
Hepatocellular cancer	Schmid et al, 2007
Melanoma	Nash et al, 2007
Papillary thyroid cancer	Ringel et al, 2002

Table 10. Types of cancer affected by KiSS-1/gpr-54 expression. Table listing the types of cancer which have been reported to be associated with regulation by KiSS-1 and gpr-54.

5.2. Results

5.2.1. Kisspeptin-10 inhibits migration of CHO cells stably expressing human gpr-54 via an ERK1/2-dependant mechanism

In the previous chapter, it was shown that migratory signalling proteins were activated in the CHO/gpr-54 cell line and it has also previously shown that kp-10 can inhibit the migration of this cell line when the receptor is stably expressed (Hori et al., 2001; Ohtaki et al., 2001). However, the molecular pathways associated with this inhibition are yet to be elucidated, the following research aims to analyse the response of the pathways to gpr-54 activation. It was firstly established that migration could be inhibited within the CHO/gpr-54 cell line using a cell migration assay. In these cells, kp-10 caused a potent dose-dependant inhibition of migration at 22 hours, with 100nM kp-10 maximally inhibiting migration by 80% at 22 hours. To prove that this was specifically due to activation of gpr-54, the gpr-54 antagonist characterised in chapter three, peptide 234 was used. 100nM peptide 234 was able to ablate the inhibition of cell migration caused by 100nM kp-10 at 22 hours, with no intrinsic effects when used alone (Fig. 83 and Table 11). Now that it was established that kp-10 and gpr-54 were able to inhibit the migration of this cell line, inhibitors were used to delineate the signalling molecules responsible for this action. 100nM kp-10 mediated inhibition of migration was blocked

when inhibitors of $G_{q/11}$, calcium, EGFR, PKC, MEK, p90rsk and GSK3β were used (Fig. 84 and Table 11). This suggests that GSK3β inhibition via ERK1/2 and p90rsk, as determined in chapter four is responsible for this effect probably through regulation of adherens junctions.

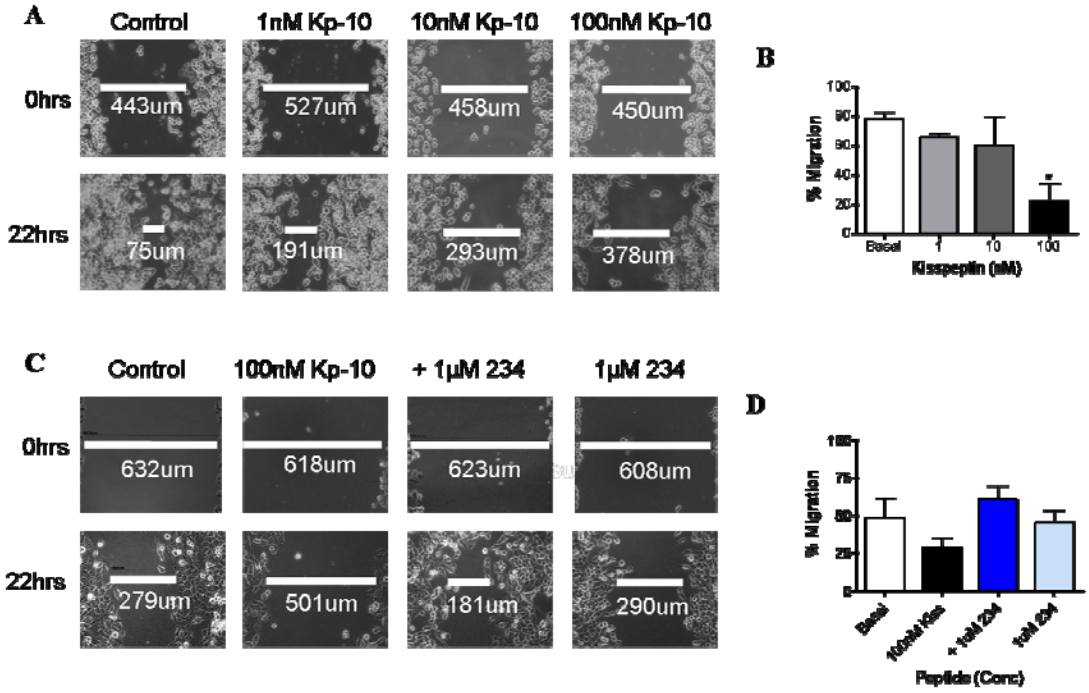

Figure 83. Kp-10 inhibits migration via gpr-54 in CHO/gpr-54 cells. (A) Kp-10 inhibits cell migration in a dose-dependant manner at 22 hours with 100nM inhibiting by 80% (n=5). (B) Quantification of cell migration assay showing dose-dependant inhibition. (C) Kp-10 inhibited migration is ablated by the gpr-54 antagonist, peptide 234 with no intrinsic effects (n=3). (D) Quantification of cell migration assay showing kp-10 mediated inhibition of migration and ablation by peptide 234.

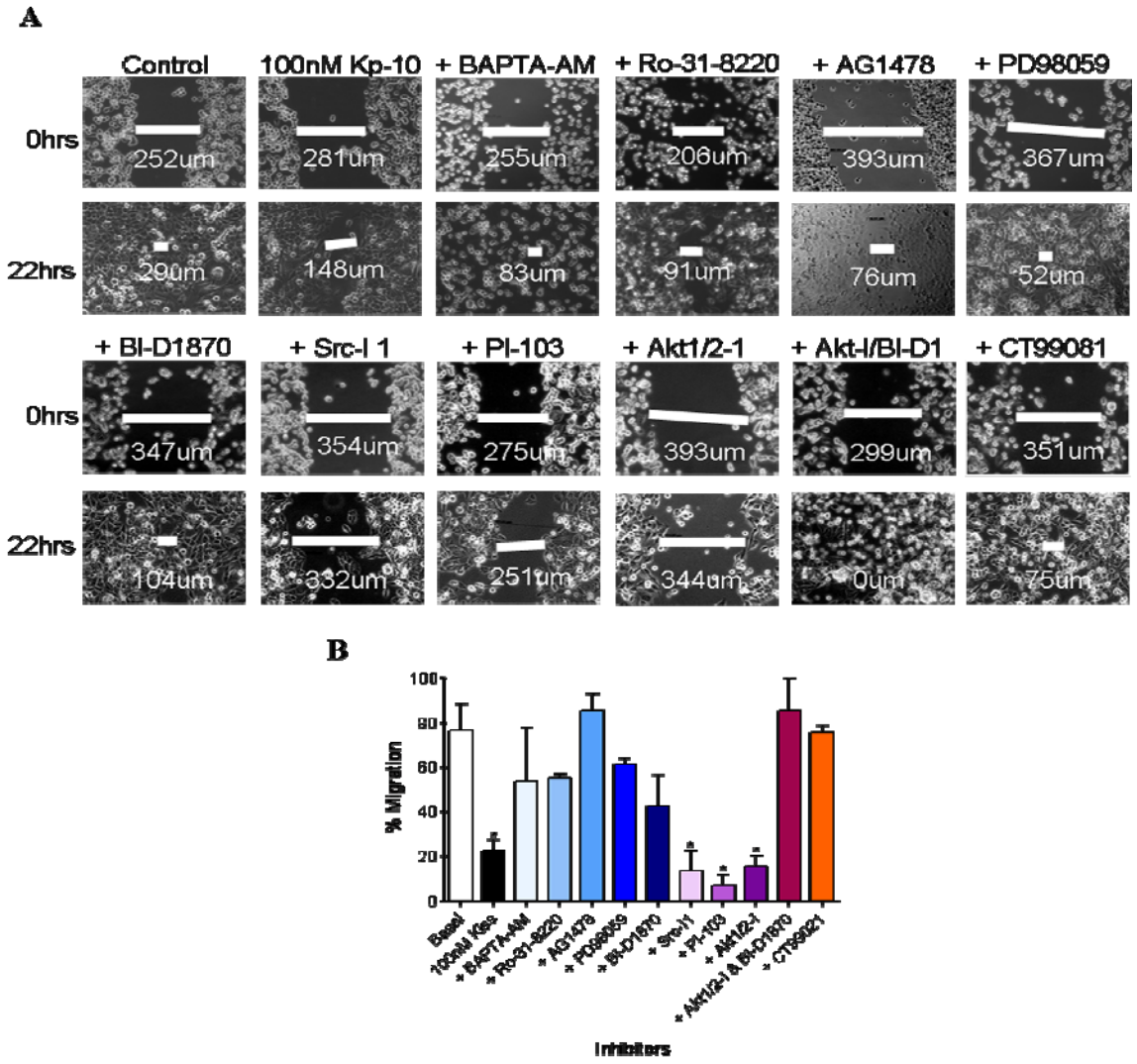

Figure 84. Kp-10 inhibits migration via an ERK1/2 dependant mechanism in CHO/gpr-54 cells. (A) Cell migration assay showing inhibition of migration by kp-10. This was ablated by 1μM BAPTA-AM (Calcium), 1μM Ro-31-8220 (PKC α, β, γ, ε), 1μM AG1478 (EGFR), 20μM PD98059 (MEK), 1μM BI-D1870 (p90rsk) and 1μM CT99021 (GSK3β) inhibitors (n=4). (B) Quantification of cell migration assay showing percentage migration and ablation by specific inhibitors.

In this pathway FAK was activated to effect MAPK activation. However, FAK can also associate with integrins at focal adhesions and kp-10 has previously been shown to

promote formation of focal adhesion complexes (Ohtaki et al., 2001). To investigate whether focal adhesions were involved in the kp-10 mediate inhibition, an Arg-Gly-Asp-Ser (RGDS) tetrapeptide was used as this is the minimum consensus peptide sequence required to disrupt integrin receptor interactions with the extracellular matrix. When an excess of RGDS is incubated within cells this displaces the extracellular region of the integrin from the ECM disrupting the integrin receptor dimer causing the destruction of the focal adhesion complex (Davidson et al., 2004). Therefore, RGDS was used to examine the influence of focal adhesions on kp-10 mediated inhibition of migration and another tetrapeptide, RGES was used as a negative control. However, the use of both RGDS and RGES did not affect the ability of kp-10 to inhibit the migration of these cells, suggesting that focal adhesions do not play a role in this action (Fig. 85).

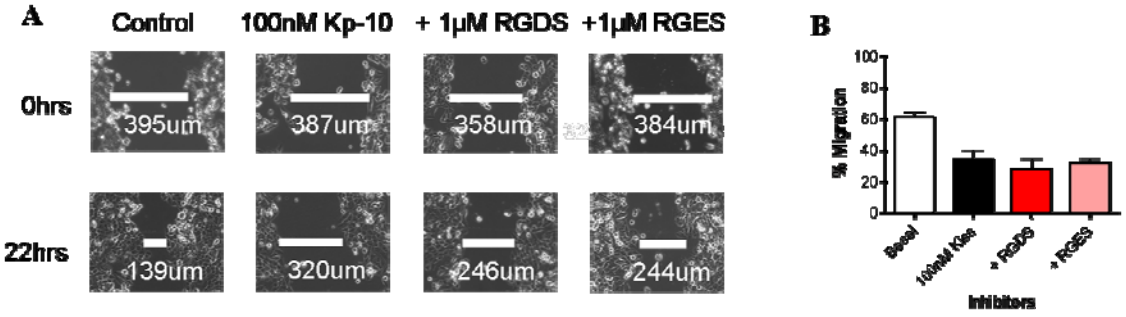

Figure 85. Integrins are not responsible for kp-10 mediated inhibition of migration. (A) Cell Migration assay assessing kp-10 mediate inhibition of migration in CHO/hgpr-54 cells. This is not ablated by the integrin disrupting tetrapeptide RGDS or the negative control tetra peptide RGES (n=3). (B) Quantification of cell migration assay showing RGDS does not disrupt the kp-10 mediated inhibition of migration in CHO/hgpr-54 cells

5.2.2. Kisspeptin-10 does not affect migration of mouse embryonic GnRH neuronal cells or human MCF-7 breast cancer cells

As GSK3β and ERK1/2 were activated in GnRH neuronal cells, it was decided to analyse whether kp-10 had any migratory effects in this cell type or if kisspeptins primary role in the GnRH neuron is the regulation of GnRH production. This was

examined using an *in vitro* cell migration assay with 1-100nM kp-10 but no inhibition of migration was seen in these cells, with relatively little migration even in the control cells throughout the 22 hour time period. It therefore appears the migratory effects of kp-10 are cell and tissue specific (Fig. 86 and Table 11).

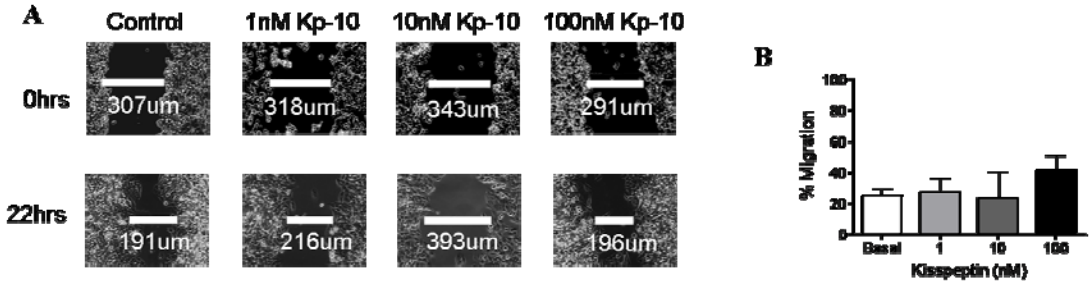

Figure 86. Kp-10 does not affect the migration of GnRH neuronal cells. (A) Cell migration assay showing no effect of kp-10 on the GT_{1-7} neuronal cell line stably expressing mouse gpr-54 (n=3). (B) Quantification of cell migration assay showing no effect on the migration of these cells.

Since kp-10 has been shown to have anti-metastatic effects in cancer cells, MCF-7 breast cancer cells were examined to explore if this was due to an inhibition of migration in response to kisspeptin. However, 100nM kp-10 could not inhibit the migration of these cells by 22 hours of migration (Fig. 87 and Table 11). Also, no effect of peptide 234 was seen as expected. Therefore it appears that inhibition of migration does not play a role in kisspeptins anti-metastatic properties in these cells.

5.2.3. Kisspeptin-10 inhibits the migration of HUVEC cells via an ERK1/2-dependant mechanism

The main migrational affects of kisspeptin reside in the placental cells where kisspeptin inhibits trophoblast invasion into the maternal vasculature. To examine the mechanism of this inhibition, human cell lines from two different areas of the placenta were examined; human umbilical vein endothelial cells (HUVECs) and immortalised first trimester extravillious trophoblast cells (HTR8SVneo).

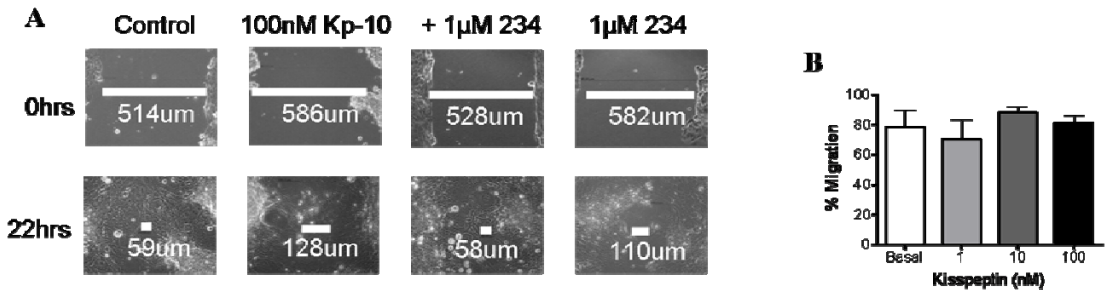

Figure 87. Kp-10 does not inhibit the migration of MCF-7 breast cancer cells. (A) 100nM kp-10 does not inhibit migration within a MCF-7 cell migration assay and peptide 234 also has no affect (n=2). (B) Quantification of cell migration assay showing no differences in percentage of migration between controls and treatments.

In the HUVEC cell line, kp-10 was able to inhibit migration in an inverse dose-dependant manner with 1nM giving a maximal inhibition of 50% at 16 hours (16 hours was used for the placental cell lines as 22 hours was to long to see an effect). Increasing the dose of kp-10 reduced the percentage of inhibition (Fig. 88 and Table 11). Again to verify that the inhibition noted was due to gpr-54 in these cells, two of the antagonists delineated in chapter three, peptides 234 and 273 were utilised. Both of these antagonists ablated the kp-10 mediated inhibition of migration in these cells, confirming the affect is mediated via gpr-54. Since, kp-10 could inhibit the migration of the HUVEC cells, inhibitors were again employed to delineate the mechanism of this inhibition. These studies revealed that inhibitors of $G_{q/11}$, Src, PKC, EGFR and ERK1/2 could ablate the inhibitory affects of kp-10 (Fig. 88 and Table 11). These results again suggest that the ERK1/2, p90rsk, GSK3β pathway to release β-catenin is also responsible for this inhibition in the placental HUVEC cells as well as in the model CHO cell line and both are via an EGF receptor transactivation mediated pathway.

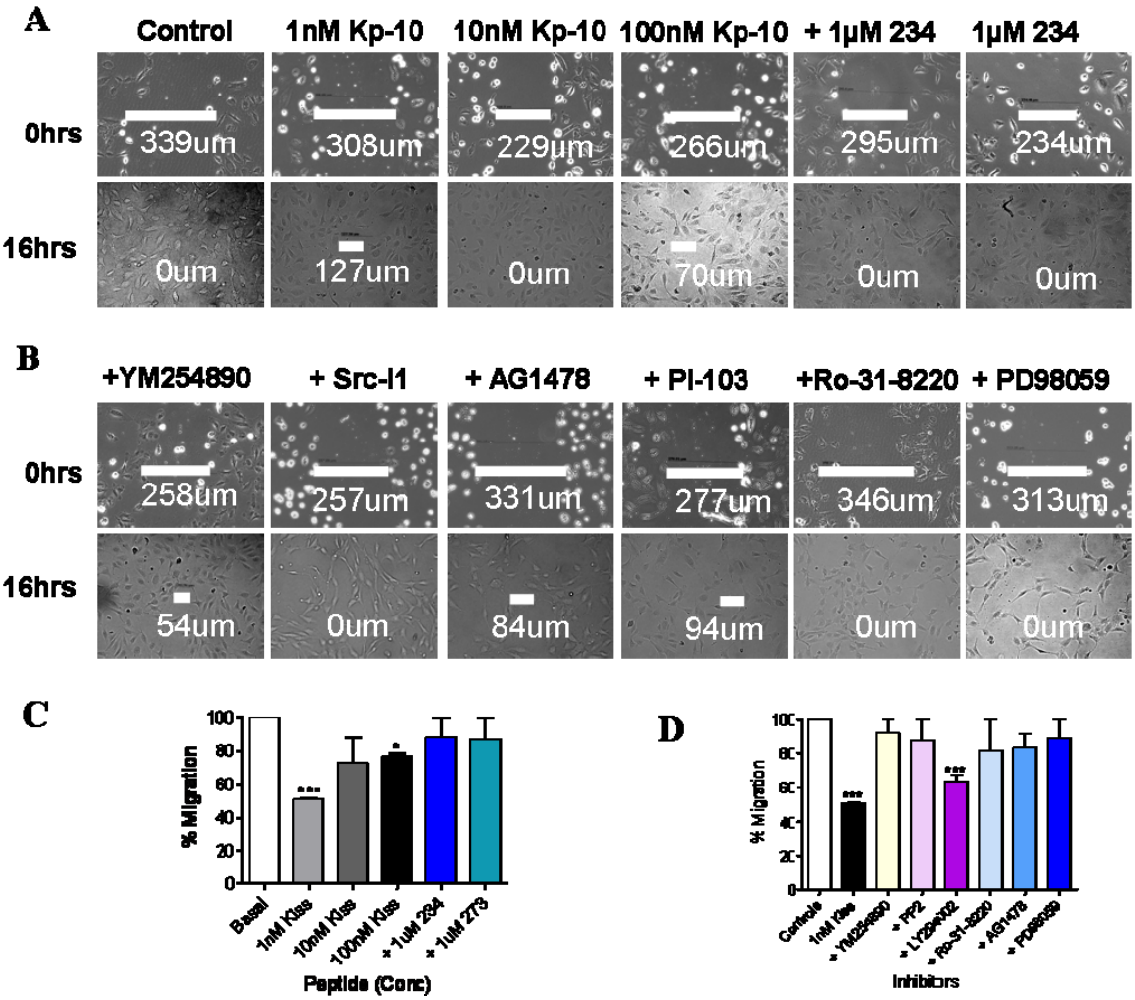

Figure 88. Kp-10 inhibits migration within HUVEC placental cells. (A) Kp-10 inhibits migration in a dose-dependant manner with 1nM kp-10 giving a maximal inhibition of 50% using a cell migration assay (n=3). (B) Kp-10 mediated inhibition is ablated with 100nM YM254890 ($G_{q/11}$), 1µM Src Inhibitor 1 (Src), 1µM AG1478 (EGFR), 1µM Ro-31-8220 (PKC α, β, γ, ε) and 20µM PD98059 (MEK) inhibitors (n=2). (C, D) Quantification of cell migration assays showing inhibition by kp-10 and ablation by selected inhibitors.

5.2.4. Kisspeptin-10 inhibits migration of HTR8SVneo trophoblast cells via an ERK1/2-dependant mechanism

Next, the first trimester extravillious trophoblast cell line, the HTR8SVneo cells were tested in the cell migration assay; these cells were derived from an area of the placenta

where gpr-54 is supposed to elicit inhibitory affects. As expected, kp-10 could inhibit the migration of these cells in a dose-dependant manner with 100nM giving a maximal inhibition of 50% (Fig. 89 and Table 11). The signalling pathways responsible for this inhibition were investigated using inhibitors. Kp-10 mediated inhibition of migration was blocked in these cells with inhibitors of $G_{q/11}$, PKC, EGFR, ERK1/2, Akt, p90rsk and GSK3β (Fig. 89 and Table 11). This is the same pathway utilised in both the CHO and HUVEC cells suggesting a common pathway is used to elicit kisspeptins migratory affects.

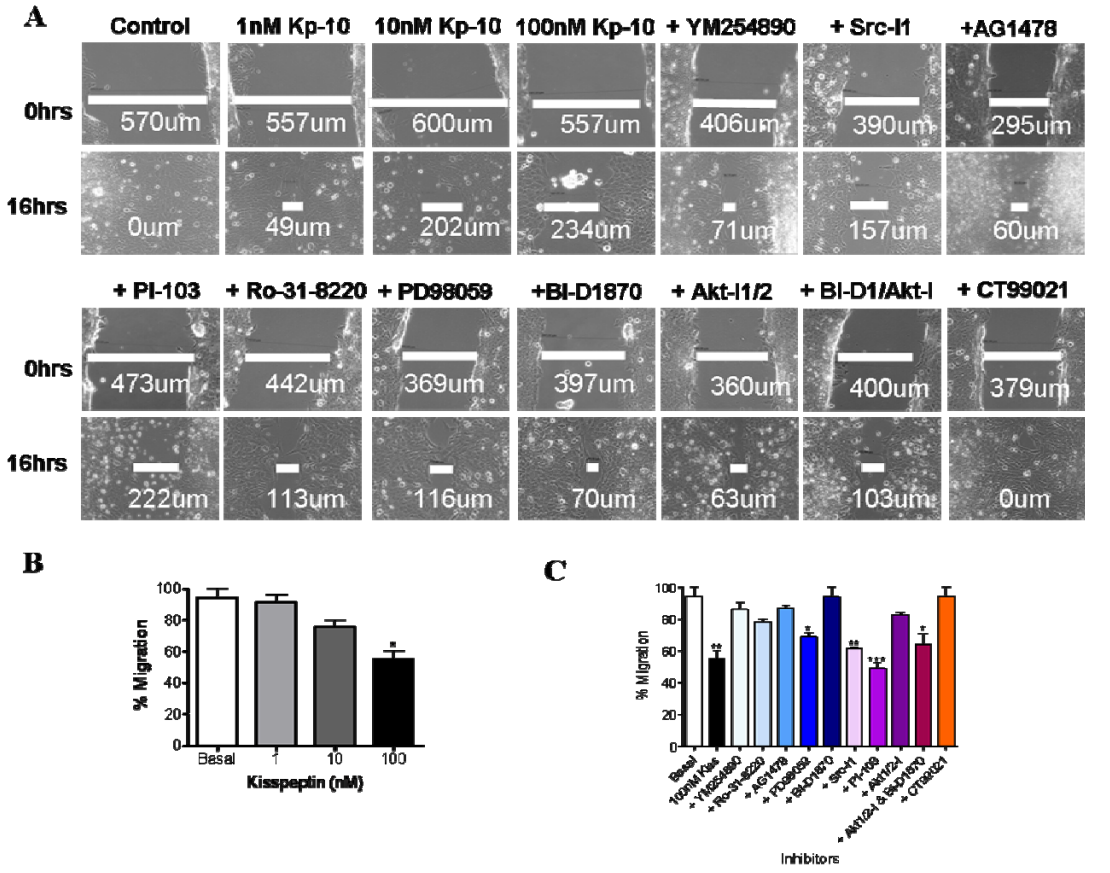

Figure 89. Kp-10 inhibits migration in trophoblast cells via an ERK1/2-dependant pathway. (A) Kp-10 inhibits the migration of HTR8SVneo trophoblast cells in a dose dependant manner with 100nM giving a maximal inhibition of 50% in a cell migration assay (n=3). This inhibition is ablated by 100nM YM254890 ($G_{q/11}$), 1μM AG1478 (EGFR), 1μM Ro-31-8220 (PKC α, β, γ, ε), 20μM PD98059 (MEK), 1μM BI-D1870 (p90rsk), 1μM Akt-I1/2 (Akt) and 1μM CT99021 (GSK3β) inhibitors (n=3). (B, C) Quantification of cell migration assays showing inhibition of kp-10 and ablation of this inhibition by select inhibitors.

Species	Cell type	Effect of Kp-10 on migration	Mecahnism
Human	CHO epithelial-like	80% inhibition at 100nM Kp-10	$G_{q/11}$, PKCε, Calcium, EGFR, ERK1/2, p90rsk, GSK3β
Mouse	GnRH neuronal-like (GT_{1-7})	No inhibition	n/a
Human	Breast cancer epithelial (MCF-7)	No inhibition	n/a
	Umbilical Vein Endothelial (HUVEC)	50% inhibition at 1nM Kp-10	$G_{q/11}$, PKCε, Src, EGFR, ERK1/2
	Placental trophoblast (HTR8SVneo)	50% inhibition at 100nM Kp-10	$G_{q/11}$, PKCε, EGFR, ERK1/2 p90rsk, Akt, GSK3β

Table 11. Summary of kp-10 mediated migrational effects in a variety of cell types. Table summarising the migrational effects mediated by kp-10. The mechanisms responsible for this inhibition are also shown.

5.3. Discussion

In the previous chapter it was shown that complex intracellular signalling pathways are mediated by kisspeptin-10 upon activation of gpr-54 in a stably transfected CHO epithelial-like cells, immortalized embryonic GnRH neuron-like cells as well as human breast cancer cells and immortalized human placental trophoblast cells. In this chapter, these pathways are revealed to be involved in kisspeptin mediated inhibition of migration in two of these cell types; CHO/gpr-54 and placental cell lines. In GnRH neurons and MCF-7 breast cancer cells no effect on migration via kp-10 and gpr-54 could be observed.

The involvement of MAPK pathways is well documented in the signalling mechanisms of GPCR receptor activation, with many other receptors also signalling via these molecules, such as GnRH-R (White et al., 2008). For the GnRH receptor the MAPK pathway has been implicated in the induction of cell death (White et al., 2008), regulation of gene transcription of gonadotropin subunits and gonadotropin release (Naor, 2009). It is therefore possibly that this more classical pathway could stimulate release of GnRH from neurons upon gpr-54 activation, which has so far been associated

with calcium fluxes (Constantin et al., 2008; Liu et al., 2008). However, here we have shown that MAPK mechanisms are also involved in the migrational effects. We can show that ERK1/2 activates p90rsk, a molecule known to be involved in the inhibition of GSK3β via phosphorylation of Ser^{9}, a glycogen synthase signaling mechanism (Torres et al., 1999). GSK3β, is usually associated with β-catenin activation in tumorigenesis/embryonic development and cell fate specification (Kim and Kimmel, 2000), but has recently been shown to be activated by GPCR signaling cascades, for example by the GnRH receptor (Gardner et al., 2007). For the GnRH receptor, GSK3β has been implicated in the accumulation of β-catenin in the nucleus to modulate gene transcription. However, this does not appear to be the mechanism for gpr-54, as Akt needs to phosphorylate β-catenin for nuclear translocation to occur, however kisspeptin has recently been shown to disrupt EGFR mediated Akt activation (Daugherty and Gottardi, 2007; Fang et al., 2007; Navenot et al., 2009). This decrease may be due to an increase of PTEN, as p38MAPK is phosphorylated by kisspeptin-10, which is needed to up regulate PTEN via transcription of AFT2, (Shen et al., 2006), though further investigation is needed. This inactivation of Akt is also supported as we have shown that GSK3β inhibition at Ser^{9}, a mechanism needed to release β-catenin is only slightly decreased by members of the Akt pathway. It seems that for gpr-54, GSK3β and β-catenin are associated with migration via p90rsk. P90rsk is known to also phosphorylate Ser^{9} of GSK3β, but causes β-catenin to be retained in the cytoplasm and associate with cadherins (Torres et al., 1999). Once β-catenin has bound cadherins it can then bind to α-catenin which is linked to the actin cytoskeleton causing formation of adherens junctions to inhibit cell movement and migration (Aberle et al., 1996; Daugherty and Gottardi, 2007). This mechanism may be critical in the inhibition of trophoblast invasion mediated by kisspeptin and gpr-54.

The inhibition of GSK3β via ERK1/2 and p90rsk appears to be important in the CHO/gpr-54 and immortalised placental trophoblast cells. In these two cell types, FAK is also regulated by kp-10 via phosphorylation at Tyr^{925}. This phosphorylation creates a SH2 binding site for the Grb2-SOS complex within FAK. As SOS is a nucleotide

exchange factor this can increase the activation of RAS to increase ERK1/2 and p90rsk phosphorylation (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996). In the previous chapter this phosphorylation of FAK was shown to be dependant on ERK1/2, p90rsk, Akt and GSK3β creating a feedback loop via FAK and Grb2 to increase ERK1/2 and p90rsk phosphorylation to further inhibit GSK3β and increase β-catenin within the cytoplasm. This would further increase the formation of adherens junctions to inhibit cell movement. Another role of FAK may be to associate with integrins to form focal adhesion complexes. FAK usually forms a complex with Src and two other proteins, paxillin and talin upon activation by phosphorylation (Li and Hua, 2008; Petit and Thiery, 2000). This complex can then interact with integrins to link the ECM to the cytoskeleton, holding adjacent cells together and allowing the assessment of stresses upon the cell. Although these results suggest that FAK via integrins play a role in this migrational affect, the use of an integrin disrupting tetrapeptide, RGDS, shows no effect. When RGDS is added to cell cultures it can displace integrins but cannot bind the ECM disrupting focal adhesions (Davidson et al., 2004), as the use of this peptide had no affect on kp-10 mediated inhibition of migration it appears that focal adhesions are not responsible for this affect or that their loss can be compensated for. Therefore, the main role of FAK in this pathway may be to enhance the MAPK signalling pathway through ERK1/2.

Kisspeptin-mediated activation of NF-kappa B has also been implicated in the inhibition of trophoblast invasion via down regulation of NF-kappa B binding to the promoter of MMP-9. This reduces expression of MMP-9 leading to inhibition of ECM degradation and trophoblast invasion (Yan et al., 2001). Activation of NFκB by phosphorylation via kp-10 was shown in the previous chapter within all cell types and this was shown to be dependant on PKC activation only. However, its importance in kp-10 mediated inhibition of migration is yet to be assessed due to a lack of specific and potent inhibitors for this pathway.

The above results show that kp-10 is a potent inhibitor of migration in selected cell types such as placental cells, where this action may prove advantageous for restricting the amount of trophoblast invasion during placentation. However, the lack of effect on the migration of GnRH neuronal cells, GT_{1-7}, suggests that the actions and signalling mechanisms mediated by kp-10 and gpr-54 are cell-type specific rather than universal. This is interesting as similar proteins, although only a select few are increased by kp-10 in these cells, giving the potential for similar actions at both sites. Therefore, kp-10 must be able to differentially regulate the same molecules for diverse purposes; however the function of these pathways in the GnRH neuron is still to be examined. This cell type specific signalling seen for kp-10 and gpr-54 may arise via a multitude of mechanism within the different cells. The differences may be the result of receptor expression levels, signalling dynamics, differences in components of the signalling apparatus or due to differential post-translational modifications of the receptor. This cell-type specific signalling is further confirmed by the lack of inhibition on the migration of MCF-7 breast cancer cells, as kp-10 has been shown to inhibit metastasis in this type of cancer and again similar pathways are activated. This suggests that different mechanisms are utilized by kp-10 to inhibit cancer metastasis and trophoblast invasion, further confirming the cell type specificity of kisspeptins actions.

In summary, in placental cells kp-10 activates a GSK3β dependant mechanism to release β-catenin into the cytoplasm to associate with cadherins causing an inhibition of the migration of these cell lines. This pathway is then further amplified via a FAK mediated feedback loop to increase MAPK signalling enhancing p90rsk mediated GSK3β inhibition. To further confirm this pathway, the affect of kp-10 on cadherins and examination of β-catenin's association with these upon kp-10 stimulation needs to be investigated. Also the effects of the inhibitors alone on the migration of each cell type also have to be assessed. However, it is clear that this mechanism is essential for these actions both in extravillious trophoblast and umbilical cord cells within the placenta, but primary tissue and *in vivo* effects still need to be examined.

Chapter Six

Discussion

6.0. Summary

The research presented within this thesis has identified important residues within kisspeptin-10 that facilitate binding to (Asn^2, Trp^3, Phe^6, Arg^9, Phe^{10}) and activation of (Tyr^1, Leu^8) gpr-54. Four novel potent synthetic peptide kisspeptin antagonists were produced. One antagonist was used *in vivo* to confirm the role of kisspeptin in the control of gonadotropin secretion, mediation of steroid-hormone feedback, the pre-ovulatory LH surge and the onset of puberty.

This thesis also includes analyses of the intracellular signalling pathways activated following gpr-54 activation. The role of these pathways in kisspeptin-mediated inhibition of cell migration was studied in a variety of cell types. The results showed that gpr-54 activation affected MAPK, GSK3β, NFκB and FAK signalling and that these signals create a feedback loop to enhance GSK3β inhibition by Ser^9 phosphorylation resulting in release of β-catenin into the cytoplasm in epithelial-like CHO and human placental trophoblast cells. This pathway was shown to be critically important for kisspeptin-mediated inhibition of the *in vitro* migration of these cells and HUVEC cells. In contrast, this pathway was not activated in GnRH neuronal cells or a human breast cancer cell line.

6.1. Summary of Chapter Three

Since kisspeptin was shown to be a potent regulator of the HPG axis with functions in steroid hormone feedback and the initiation of puberty, different studies have emerged to try to delineate the mechanism of this regulation. However, evidence for the functions of kisspeptin in the HPG axis has been indirect due to a lack of potent antagonists. Therefore, in this chapter a structure-function study was undertaken using intuitive changes to the kisspeptin-10 sequence, to determine which residues in synthetic peptides are involved in binding and activating gpr-54. Cell culture studies using these peptides enabled screening for potent agonists and antagonists.

These studies identified five residues in kisspeptin-10 involved in binding to gpr-54. Two residues were shown to be involved in receptor activation and four potent kisspeptin antagonists were identified (Fig. 90). Firstly, Phe^{6}, Trp^{9} and Phe^{10} were shown to be critical to receptor binding as substitution of each of these residues abolished binding to gpr-54. This occurred even when the side chains were moved out of position via substitution with D-amino acid isomers. Alterations to two further residues, Asn^{2} and Trp^{3}, also significantly decreased binding to the receptor when substituted with alanine, suggesting that the side chains were important either for ligand-receptor interactions or structural conformation of the peptide. Two residues were also shown to be involved in activation of the receptor, substitution of Leu^{8} ablated stimulation of IP release and increased receptor antagonism and similar results were obtained upon substitution of Tyr^{1} (Fig. 90). Finally, these studies produced four potent antagonists, all of which possessed Tyr^{8} to create steric hindrance within the C-terminal region combined with small, flexible side chains (glycine or D-serine) in place of Ser^{5}. This suggests that antagonists require rigidity in the C-terminal region and flexibility in the N-terminal region. Antagonism was further enhanced by substitutions at Tyr^{1}, with alanine being the most potent substitution (peptide 234; Fig. 90).

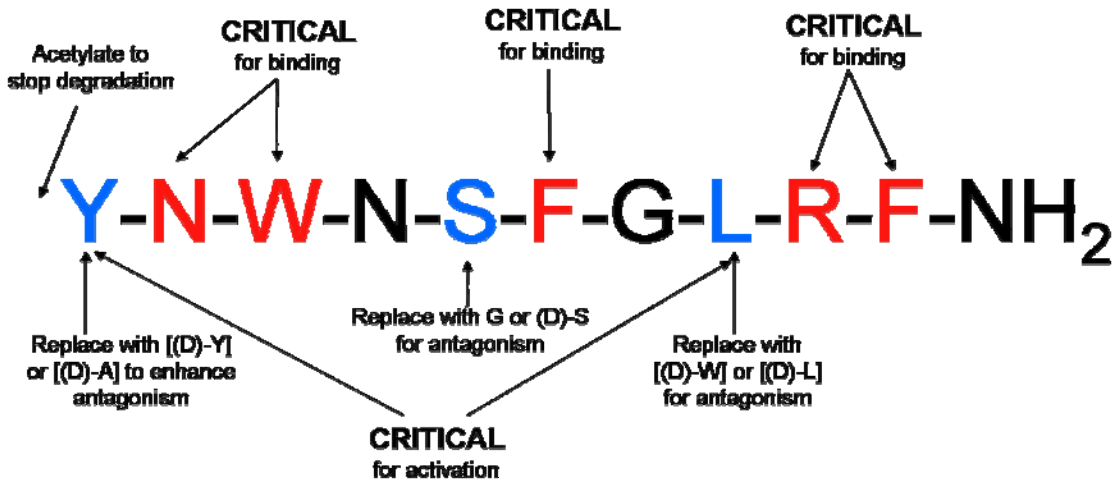

Figure 90. Schematic diagram of important kisspeptin-10 residues. Residues that are important for receptor binding (red) and receptor activation (blue) within kp-10 are highlighted.

Peptide 234 was selected to be tested for effects *in vivo* to assess the role of kisspeptin in the regulation of gonadotropin secretion. Firstly, peptide 234 was shown to inhibit firing of the GnRH neuron in mice and pulsatile GnRH secretion in primates, confirming that kisspeptin regulates the secretion of GnRH. Peptide 234 could also ablate the post castration rise of LH in rodents and LH pulse amplitude in OVX ewes, but had no effect on basal LH levels. This suggests that basal and pulsatile GnRH/LH may be regulated by separate pathways within the brain and also confirms the role of kisspeptin in negative steroid feedback. Application of peptide 234 also confirmed a role for kisspeptin in the pre-ovulatory LH surge in rats as this was completely inhibited and in the onset of puberty, as peptide 234 delayed vaginal opening in rats. Thus, peptide 234 is a potent *in vivo* kisspeptin antagonist and is a useful tool to investigate the actions of kisspeptin in a variety of species. Kisspeptin antagonists may also be useful as medicines for conditions involving elevated levels of gonadotropins such as PCOS and precocious puberty or even as a novel contraceptive.

6.2. Summary of Chapter Four

To date the intracellular signalling pathways mediated by kisspeptin and gpr-54 have remained relatively uncharacterized. A select number of studies have focused on the intracellular mechanisms of gpr-54 signalling mainly in cancer tissue. Therefore, in this chapter studies were undertaken to elucidate the intracellular pathways mediated by kisspeptin-10 in a variety of cell types. The aim was to look for cell-specific activation of signalling.

The studies within the chapter used four main cell types; epithelial-like CHO cells stably expressing human gpr-54, mouse $GT_{1\text{-}7}$, GnRH neuron-like cells, human MCF-7 breast cancer cells and human HTR8SVneo placental trophoblast cells. Similar signalling pathways were shown to be active in each cell type with some mechanistic differences. In the CHO, MCF-7 and HTR8SVneo cell lines, kisspeptin-10 mediated effects on

MAPK, GSK3β, FAK and NFκB signalling pathways. A major pathway activated in these cells was activation of ERK1/2 enhanced p90rsk-mediated inhibition of GSK3β via Ser^{9} phosphorylation. This caused release of β-catenin from GSK3β and resulted in phosphorylated of FAK at Tyr^{925}, which in turn increased phosphorylation of ERK1/2 (Fig. 91). The main differences between these cell types are the initial mode of ERK1/2 phosphorylation. In CHO cells, only EGF transactivation is needed for the MAPK pathway to be activated, whereas in the HTR8SVneo cells PKCε and transactivation of the EGF receptor are required. This suggests that kisspeptin may regulate the migration of these cells via modulation of adherens junctions.

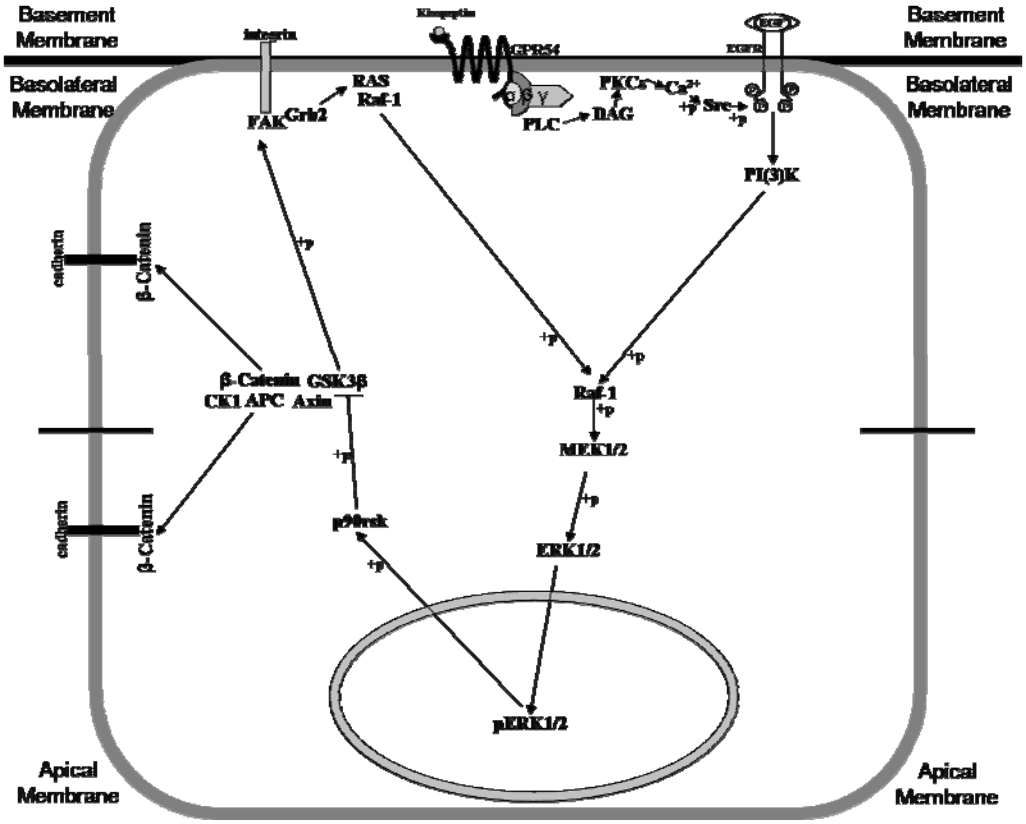

Figure 91. Schematic diagram of the ERK1/2-mediated signalling pathway activated by gpr-54. Potential feedback loop involving ERK1/2, p90rsk, GSK3β and FAK-Grb2 to release β-catenin into the cytoplasm.

As for the GT_{1-7} cells, signalling appeared less diverse when activated by kisspeptin/gpr-54. Only ERK1/2, GSK3β and NFκB were modulated by gpr-54; all of which appear to rely on EGF receptor transactivation. Although these are similar to the pathways activated in the other cell types, the consequences of signalling may be different as adherens junctions are not found in neuronal cells. No regulation of β-catenin was observed.

6.3. Summary of Chapter Five

Since some of the intracellular signalling pathways mediated by kisspeptin and gpr-54 had been delineated in the previous chapter, the next step was to investigate the function of this signalling. As many of these pathways have previously been shown to affect cell migration and kisspeptin can inhibit this process, cell migration was the functional output chosen. Therefore, in this chapter kisspeptin was tested for its ability to inhibit the migration of the cell types studied in chapter four and then inhibitors were used to test the involvement of the delineated pathways.

From these studies, it was determined that kisspeptin could inhibit the migration of epithelial-like CHO cells, HUVEC cells and placental trophoblast cells but had no effect on the migration of MCF-7 breast cancer cells or GT_{1-7} neuronal cells. In the CHO and trophoblast cells, this inhibition was dose-dependant with 100nM kisspeptin-10 inhibiting migration by 80% and 50%, respectively at 22 or 16 hours. In HUVEC cells, the inhibition seemed to follow an inverse dose-response with 1nM inhibiting migration by 50% and this gradually decreased as the dose increased. However, it was clear that kisspeptin-10 could inhibit the migration of these cells and therefore inhibitors were used to elucidate the pathways responsible for this inhibition. In all three cell types, the ERK1/2-mediated pathway delineated in chapter three was shown to be responsible for effects on migration. This effect probably involves the formation of junctions between cells such as adherens junctions involving β-catenin released via GSK3β inhibition. One

consequence may be the alteration of MMP-9 production via NFκB; however, due to a lack of specific inhibitors for this pathway, further studies would be required to determine these possibilities. Focal adhesions had also been hypothesized to be involved in this inhibition; however the use of RGDS tetrapeptides to disrupt the focal adhesion complex did not affect kisspeptin-10 mediated inhibition of cell migration suggesting this is not the case.

6.4. Future Work

6.4.1. Kisspeptin antagonists in vitro

Kisspeptin is a pivotal component of the HPG axis, regulating the onset of puberty, gonadotropin secretion and the pre-ovulatory LH surge. However, due to the indirect methods used for examining these functions to date, this thesis elucidated four potent kisspeptin antagonists, each possessing the potential for direct assessment of the functions of kisspeptin and gpr-54. These antagonists still need further characterization *in vitro,* for example to evaluate the on-rate and off-rate of each analogue, or to calculate the half life of each analogue in *in vivo* studies.

However, there is a problem with the binding assay. The ^{125}I-radiolabelled kisspeptin exhibits non-specific binding to plastic, making interpretation of whole cell binding data difficult. To try to resolve this problem, coating of the plate with matrigel or PLL may reduce the interactions made with the plastic. Also, pre-incubation of the plates with tryptophan residues, which should hydrophobically interact with the plastic, may reduce the capacity for kisspeptin interactions allowing more binding to just gpr-54. Another problem encountered within these studies recently, is a reduction in antagonism of kisspeptin-10 stimulated IP production within the current assay used and this may be due to changes within the analogues or the assay utilised. Therefore, varying the incubation periods for each may improve the antagonism, for example, the antagonists may be

incubated for longer or kisspeptin for shorter time periods. Another way to change these effects may be to vary the temperature as temperature is known to be a vital factor for on/off rates. There is also a possibility that the analogues are forming non-covalent complexes when dissolved in propylene glycol and preliminary studies may have had no effect since the kisspeptin was still dissolved in propylene glycol when mixed with the analogues. Therefore, studies examining the use of different solvents to dissolve both the analogues and kisspeptin may be beneficial.

Once the assay conditions are improved, further studies may be undertaken to carefully assess the on-rate and off-rate of kisspeptin and each analogue, to give an indication of the peptides half-life. This would be done via extensive binding studies using a single concentration of labelled kisspeptin alone over a long time course. Once these rates were known for the four antagonist, modifications could then be made to each to try and improve their half life and IC_{50}. The new analogues would then need to be extensively tested *in vitro* to assess their ability to bind to and activate gpr-54, to test for any cross reactions with other RFamide receptors and to assess their ability to antagonize at gpr-54. These studies would be beneficial to the *in vivo* studies as it should decrease the dosing for each species and the length of stimulation used.

However, if the inositol phosphate assay cannot be improved then other assays would need to be utilized. Two other assays maybe used, the FLIPR calcium 4 assay which could be modified to increase the resulting antagonism observed or an SRE-luciferase reporter assay using the LβT2/gpr-54 cells, where stimulation of SRE-luciferase with kisspeptin has been demonstrated. Therefore, this assay could be used to examine the antagonism of the analogues within these cells. Once the antagonists are refined, they could then be further utilized *in vitro* to assess signalling pathways or to examine the role of kisspeptin within GnRH neuronal cell lines on GnRH secretion from the cells.

6.4.2. Kisspeptin antagonists in vivo

Since antagonists could potentially show direct effects of kisspeptin in the HPG axis, *in vivo* testing is essential. Within this thesis one of the antagonists, peptide 234 was extensively tested *in vivo* and confirmed effects for kisspeptin in gonadotropin regulation, the timing of puberty onset and regulation of the pre-ovulatory LH surge. However, as all of these tests used central delivery, peptide 234 has to be tested for antagonism when delivered peripherally. Also, the three other antagonists from this thesis also have to be tested for their effects *in vivo* both via central and peripheral delivery. To do this, all of the antagonists could be compared using one assay looking at the effect of each on LH secretion in the rodent, to compare the potency and half-life of the analogues. This should also help with decisions about modifications to the analogues as discussed above as when analogues are exposed to *in vivo* settings other factors come into play. These include clearance rate, peptidases and the blood-brain barrier. These may affect the analogues in ways that are unforeseeable *in vitro*. Next, any improved analogues developed via these modifications, would again need to undergo *in vivo* tests to assess the effects on the HPG axis. Also as some observations such as pubertal effects have been studied in rodents, similar experiments could be undertaken in primates, which could give results that are more comparable to the human.

Once the most potent antagonist has been evaluated both *in vitro* and *in vivo*, this peptide could then be purified for use in clinical trials. Firstly, the antagonist could be tested alone in these trials, to test if it has any effects on basal secretion of LH in the human or it could be given in conjunction with kisspeptin to assess its antagonistic effects. If the antagonist is successful in the human setting, it could then be tested to see if it can inhibit the female pre-ovulatory surge of LH. This would assess if the antagonist would be useful as an alternative contraceptive, which can inhibit ovulation without affecting basal levels of gonadotropins and steroids. Another use for the antagonist that could be tested in these trials is as a therapeutic for PCOS. In PCOS, gonadotropin levels are high causing cysts to form in the ovary (Blank et al., 2006). The

antagonists may be able to suppress the gonadotropin secretion to basal levels which should cause the cysts to decreases and may return fertility to the patient. However, this would need to be tested in an animal model of PCOS before human trials could proceed. Finally, the antagonist may also be useful for inhibiting the onset of puberty in children undergoing precocious puberty.

As well as being useful for delineating kisspeptins function in the HPG axis, another setting for *in vivo* trials with the antagonists may be the placenta. As has been shown in this thesis, kisspeptin can inhibit the migration of cells including a placental trophoblast cell lines. *KiSS-1* and *gpr-54* mRNA have also been shown to be present within the human and rat placenta and *KiSS-1* mRNA is known to be up regulated in the pathological condition pre-eclampsia (Bilban et al., 2004; Hiden et al., 2007; Terao et al., 2004; Zhang et al., 2006). In pre-eclampsia, the trophoblast cells do not completely migrate into the maternal decidua and spiral artery remodelling is insufficient. The lack of migration into the decidua has been hypothesized to be due to an overproduction of kisspeptin (Sankaralingam et al., 2006). Therefore, using animal models with normal or pre-eclampsia-like placentas, the antagonist could be used to assess kisspeptins role in trophoblast invasion if given directly into the placental region and effects, if any, on the remodelling of the spiral arteries may also be assessed. This would directly evaluate kisspeptins role in this event and may provide a mechanism for increasing the amount of invasion within a pre-eclamptic placenta.

6.4.3. Kisspeptin signalling mechanisms in GnRH neuronal cells

Within this thesis, studies were also undertaken to delineate the intracellular signalling mechanisms mediated by kisspeptin in a variety of cell types. One of the cell types used was an immortalized GnRH neuronal cell line called GT_{1-7}. In these cells, only three of the proteins tested were mediated by kisspeptin; these were ERK1/2, GSK3β and NFκB. Although these proteins were also activated in other cell types, the function of these proteins in the GT_{1-7} cells seems to be different as no inhibition of migration occurs in

these cells when stimulated with kisspeptin and no release of β-catenin can be observed. However, further studies with new inhibitors may help establish a functional pathway for GSK3β in these cells. Still, other studies are needed to establish the role of these molecules within the GnRH neuron.

The obvious role for these proteins when activated by kisspeptin is to regulate the secretion of GnRH from the hypothalamus, as this is kisspeptins main role within the HPG axis (Gottsch et al., 2004; Messager et al., 2005; Popa et al., 2008). However, as secretion of GnRH cannot be detected from the $GT_{1\text{-}7}$ cells used in this thesis, other cells would need to be sourced in order to evaluate the roles of these proteins in kisspeptin-mediated secretion of GnRH *in vitro*. Other cell types that could be utilized for these studies would be Gnv3, Gn11, GTI-t or NLT neuronal cell lines reported to secrete GnRH. The first step for each cell line would be to screen for endogenous receptor and then create stable cell lines if needed. These cell lines could then be tested for kisspeptins ability to cause secretion of GnRH into the medium. If this could be established for one of the above cell lines, and the same proteins could be shown to be mediated by kisspeptin then inhibitors for these proteins could be used to examine their effects on GnRH secretion.

Another plausible function of these proteins is to regulate gene transcription within the nucleus, therefore quantitative RT-PCR could be performed for known gene targets of each protein to establish if they are mediated by kisspeptin in the GnRH neuron. Each protein can regulate a variety of genes within the nucleus; ERK1/2 has roles in the regulation of c-jun, c-myc, NFAT and fra-1, which in turn can induce cell differentiation, apoptosis or proliferation (Anjum and Blenis, 2008; Boutros et al., 2008). NFκB can also regulate gene transcription within the nucleus, to up regulate MMP-2, MMP-9, IκBα and cyclinD1 among others again promote cell differentiation and proliferation (Ghosh and Hayden, 2008; Yan et al., 2001).

6.4.4. Kisspeptin signalling mechanisms in CHO and placental cells

As well as examining kisspeptins signalling events within the GnRH neuron, two other cell types were used; epithelial-like CHO cells stably expressing human gpr-54 were used as a model cell line and immortalized human HTR8SVneo placental trophoblast cells were used to examine the signalling pathways involved in kisspeptin-mediated inhibition of trophoblast invasion. The pathways found in each cell type were very similar, with only slight differences. Both mediated an ERK1/2-p90rsk-GSK3β-βcatenin-FAK feedback loop with separate regulation of NFκB. However further experiments are still needed to clarify this pathway and to investigate if it can regulate gene transcription as well as mediate migrational effects.

To fully clarify that this is the active pathway in the HTR8SVneo cells further inhibitors for Akt, p90rsk and GSK3β could be used, as has been shown within thesis for the CHO cells. Also the inhibitors within this thesis have only been used in conjunction with kisspeptin and therefore need to be individually tested alone on each cell type to test if any of the effects seen are independent of kisspeptin stimulation. Once the pathway is clarified in both cell types, further investigation into the role of β-catenin would be merited. The studies within this thesis have only shown accumulation of β-catenin in the non-nuclear fraction; therefore further elaboration could be undertaken to establish the role of β-catenin after this accumulation. One way this could be examined is to look at the phosphorylation status of β-catenin upon release, as Akt is known to phosphorylate β-catenin at Ser^{552} to facilitate nuclear translocation and dissociation from adherens junctions (Fang et al., 2007). Therefore, investigation of the phosphorylation status of this residue may clarify whether β-catenin associates with adherens junctions or regulates gene transcription upon kisspeptin stimulation (Gooding et al., 2004; Moon et al., 2004). Another study that could be done to examine this would be to investigate the regulation of cadherins by kisspeptin. This could be done using microscopy to visualize whether kisspeptin stimulation causes cadherins to form junctions and if β-catenin associates with these or via western blots to examine if E-cadherins are either up

regulated by kisspeptin or activated via phosphorylation at $Ser^{684/686/692}$ (Gooding et al., 2004).

Another role of the pathways mediated by kisspeptin in these cells may be the regulation of gene transcription. Beta-catenin is known to bind to and activate the transcription factors, TCF/Lef by recruiting co-factors to the complex. For, example PP2A can regulate β-catenin binding to the co-factors CBP and p300 to regulate cell growth vs. cell differentiation (Daugherty and Gottardi, 2007). This could be examined using a TCF reporter construct or quantitative RT-PCR to examine regulation by kisspeptin and would further examine β-catenin's role in this pathway. ERK1/2 is also known to regulate gene transcription via activation of transcription factors in the nucleus to up regulate NFAT, c-jun and fra-1 among others, causing effects on cell differentiation, proliferation and apoptosis. Kisspeptin also phosphorylates NFκB, which is a known mediator for transcription of adhesion molecules such as MMPs. However transcription of MMPs from these two cell types would not be in line with kisspeptins inhibitory mechanism as MMPs degrade the ECM to allow cell migration (Yan et al., 2001). Therefore, studies could be undertaken to see the effect of kisspeptin on MMPs within these two cell types and inhibitors could be used to examine if NFκB is responsible or whether NFκB is playing a different role in these cells.

6.4.5. Kisspeptin migrational effects in CHO and placental cells

Studies within this thesis also mapped the involvement of the delineated signalling mechanism in cell migration within these two cells lines. This was achieved by utilizing inhibitors within a cell migration assay in conjunction with kp-10. However, the inhibitors used still need to be tested alone within this assay to assess if any of the effects observed are independent of kisspeptin. The assay used within this thesis was a scratch assay, where a scratch is made within a monolayer of cells and the migration into that scratch over time is measured. Therefore, to confirm the effects seen in this assay and the signalling mechanism delineated, these experiments could be tested using well

inserts. In this assay, the insert would be placed inside a 12-well plate and the cells grown on the insert and then kisspeptin with or without inhibitors would be added to the media and the migration through the insert would be assessed. It may also be useful to assess the affect that kisspeptin has on proliferation and differentiation within these two cell types, to confirm the results obtained are mainly due to migration rather than proliferation or differentiation.

Primary cell cultures or placental tissue could also be used to look at the migrational effects of these pathways in a more physiologically relevant setting. The use of primary cell cultures of trophoblasts would allow the evaluation of this process in unaltered cells and these could be assessed in the same manner as the immortalised trophoblast cell line. Tissue samples could also be used to assess the overall effect of kisspeptin within the placenta as the cell-cell contacts would be kept in place and the overall cellular structure of the placenta would be preserved. This may be useful as patients with mutations to gpr-54, have normal placentation during pregnancy suggesting that kisspeptin is either not involved or that it can be compensated for (Pallais et al., 2006), therefore the use of placental tissue may be able to assess the degree of involvement that kisspeptin has in this process. However, the mechanism for this examination would need further investigation.

6.5. Conclusions

The research presented within this thesis highlights the potential for direct examination of kisspeptins functions within the HPG axis and other tissues through the development of the first potent kisspeptin antagonists. It also facilitates the creation of further antagonists and agonists through the delineation of residues important for receptor binding and activation. Furthermore, these studies are the first to show regulation of GSK3β and β-catenin via an ERK1/2-p90rsk feedback loop in response to kisspeptin stimulation. This signalling pathway may be critical for kisspeptin to inhibit the migration of trophoblast cells within the placenta.

Chapter Seven

References

7.0. References

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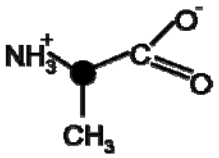
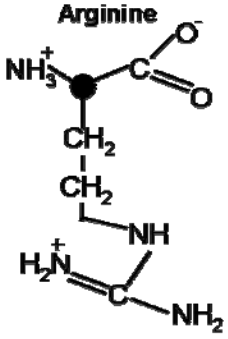
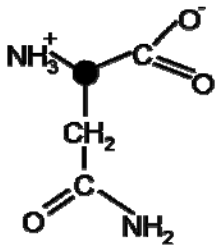
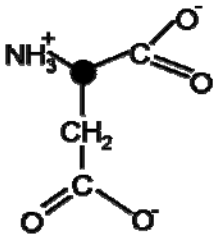
Chapter Eight

Appendices

8.0. Appendix A: Abbreviations, Charge and Structures of Amino Acids

Amino Acid	Three letter code	One letter code	Charge
Alanine	Ala	A	Non-Polar
Arginine	Arg	R	Basic
Asparagine	Asn	N	Polar
Aspartic Acid	Asp	D	Acidic
Cysteine	Cys	C	Polar
Glutamine	Gln	Q	Polar
Glutamic Acid	Glu	E	Acidic
Glycine	Gly	G	Non-Polar
Histidine	His	H	Basic
Isoleucine	Iso	I	Non-Polar
Leucine	Leu	L	Non-Polar
Lysine	Lys	K	Basic
Methionine	Met	M	Non-Polar
Phenylalanine	Phe	F	Non-Polar
Proline	Pro	P	Non-Polar
Serine	Ser	S	Polar
Threonine	Thr	T	Polar
Tryptophan	Trp	W	Polar
Tyrosine	Tyr	Y	Polar
Valine	Val	V	Non-Polar

For amino acid structures please see overleaf.

Alanine	Arginine	Asparagine	Aspartic Acid
			
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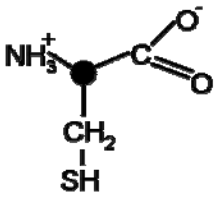	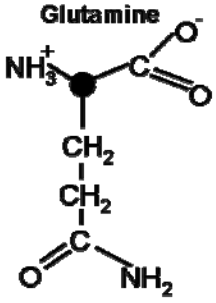	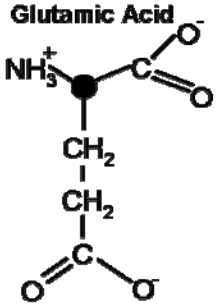	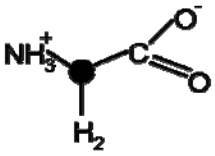
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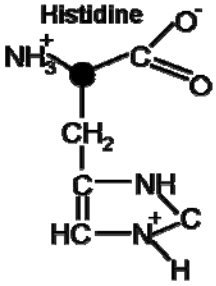
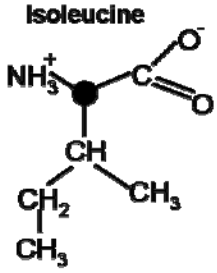
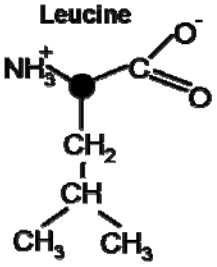
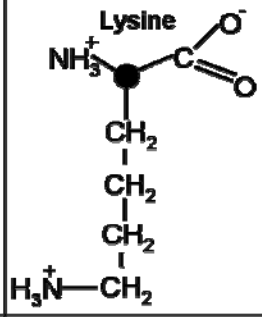
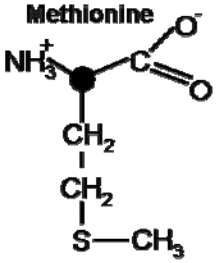
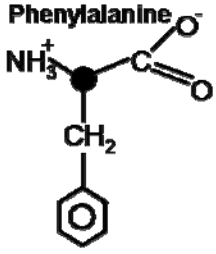
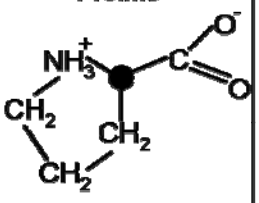
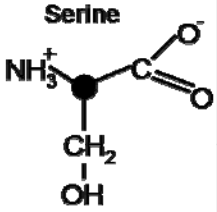
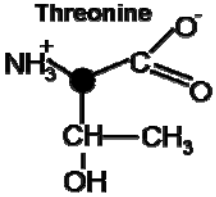
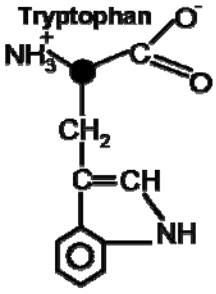
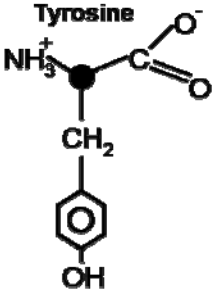
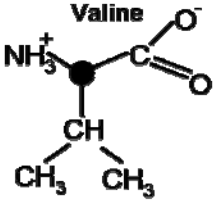
			
Methionine	Phenylalanine	Proline	Serine
			
Threonine	Tryptophan	Tyrosine	Valine
			

8.1. Appendix B: Abbreviations

aCSF	artificial Cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARC	Arcuate nucleus
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under curve
AVPV	Anteroventral periventricular nucleus
BCL	B-cell CLL/lymphoma
BDNF	Brain derived neurotrophic factor
BMI	Body Mass Index
Bp	Base pairs
BSA	Bovine Serum Albinum
BW	Body weight
Ca^{2+}	Calcium
CaMK	Camodulin-dependant kinase
CBP	CREB-binding protein
CHO	Chinese Hamster Ovary
CK	Casein kinase
CKTD	C-terminal kinase domain
COX	Cyclooxygenase
DAG	Diacyl glycerol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyriboneucleic Acid
DPC	Dodecylphosphocholine

E	Embryonic Day
EC_{50}	Excitatory constant at 50% maximum
ECACC	European Collection of Cell Cultures
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EL	Extracellular loop
EPSC	Excitatory post-synaptic current
ER	Estrogen Receptor
ERK	Extracellular-Regulated Kinase
evCT	Extravillious Trophoblasts
FAK	Focal Adhesion Kinase
FasL	Fas Ligand
FAT	Focal adhesion targeting
FERM	Band 4.1, ezrin, radixin, moesin homology
FGF	Fibroblast growth factor
FISH	Fluorescent In-situ Hybridisation
FSH	Follicle Stimulating Hormone
GABA	Gamma Aminobutyric acid
GalR	Galanin Receptor
GBP	GSK3-binding protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green florescent protein
GH	Growth Hormone
GnRH	Gonadotropin Releasing Hormone
GPCR	G-protein coupled receptor
Gpr-54	G-protein coupled receptor 54
GSK3	Glycogen Synthase Kinase 3

GTP	Guanosine triphosphate
hCG	Human Chorionic Gonadotropin
HCL	Hydrochloric acid
HPG	Hypothalamic Pituitary Gonadal
HUVEC	Human Umbilical Vein Endothelial Cells
IC_{50}	Inhibitory constant at 50% maximum
icv	Intracerebroventricular
iHH	Idiopathic Hypogonadotropic Hypogonadism
IκBα	Inhibitor of NFκB
IKK	IκB kinase
IL	Intracellular loop
ip	Intraperitoneal
IP	Inositol Phosphate
IP_3	Inositol trisphosphate
iv	Intravenous
JNK	Jun N-terminally regulated Kinase
Kir	Inwardly rectifying potassium channels
Kp-10	Kisspeptin-10
Kp-13	Kisspeptin-13
Kp-54	Kisspeptin 54
LD	Long Day
LEF	Lymphoid enhancer factor
LH	Luteinising Hormone
LiCl	Lithium Chloride
LOH	Loss of hetrozygosity
LRP	Lipoprotein receptor-related protein
LSB	2x Laemmli sample buffer
Luc	Luciferase
M	Molar
MAPK	Mitogen-activated protein kinase

MAPKAP	Mitogen-activated protein kinase-activated protein kinase
MAPKK	Mitogen-activated kinase kinase
MAPKKK	Mitogen-activated kinase kinase kinase
MCIP1	Modulatory Calcineurin-interacting Protein-1
ME	Median Eminence
MEK	Mitogen-activated protein kinase kinase
MEKK	Mitogen-activated protein kinase kinase kinase
MK	MAPK specific substrate
MKP	MAPK specific phosphatase
MLC	Myosin light chain
MLK	Mixed-lineage Kinase
MMP	Matrix Metalloproteinases
MNK	MAP kinase-interacting kinase
MSK	Mitogen- and stress-activated protein kinase
NaOH	Sodium Hydroxide
NFκB	Nuclear Factor kappa B
NMR	Nuclear Magnetic Resonance
NaCl	Sodium Chloride
NFAT	Nuclear factor of activated T-cells
NPAF	Neuropeptide AF
NPFF	Neuropeptide FF
NPSF	Neuropeptide SF
NPVF	Neuropeptide VF
NTKD	N-terminal kinase domain
ORF	Open Reading Frame
OSE	Ovarian Surface Epithelium
OVX	Ovariectomised
P	Postnatal day
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline

PCOS	Polycystic Ovarian Syndrome
PeN	Periventricular Nucleus
PG	Prostaglandin
PI(3)K	Phosphoinositide 3-kinase
PIP_2	Phosphatidyl Inositol bisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
POA	Preoptic Area
PR	Progesterone receptor
PrPR	Prolactin-releasing peptide
PTEN	Phosphatase and tensin homology
PVDF	Polyvinylidene difluoride
RFRP	RFamide related peptide
RHD	Rel homology domain
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SAPK	Stress-activated protein kinases
SD	Short Day
SDF-1	Stromal Derived Factor 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate -polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SH2/3	Src homology 2/3 domains
SP1	Specificity Protein 1
ST	Syncytiotrophoblast
TAD	Transactivation domain
TBS-T	Tris-Buffered Saline Tween-20

TCF	T-cell factor
TGF	Transforming growth factor
TM	Transmembrane domain
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRPC	Transient receptor potential canonical
TTX	Tetrodotoxin
vCT	Villous Cytotrophoblast
VO	Vaginal opening
WT	Wild type

8.2. Publications

The majority of the research in chapter three has been published in the Journal of Neuroscience:

Roseweir A.K., Kauffman A.S., Smith J.T., Guerriero K.A., Morgan K., Pielecka-Fortuna J., Pineda R., Gottsch M.L., Tena-Sempere M., Moenter S.M., Terasawa E., Clarke I.J., Steiner R.A., and Millar R.P., *Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation* J Neuroscience, 2009. **29**(12): p 3920-3929.

Part of this research was also published as a first author review in Human Reproduction Update and a third author review in Reproduction:

Roseweir A.K. and Millar R.P., *The role of kisspeptins in the control of gonadotropin secretion* Human Reproduction Update, 2009. **15**(2): p203-212.

Reynolds R.M., Logie J.J., **Roseweir A.K**., McKnight A.J. and Millar R.P., *A Role for kisspeptin in pregnancy: facts and speculations* Reproduction, 2009. **138** (1): p1-7.